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ELEVATION OF BIOTIN ACTIVITY IN THE SERUM OF ESTROGEN TREATED CHICKS; RELATIONSHIP TO HORMONE-INDUCED AVIDIN FORMATION IN THE OVIDUCT

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CONSIDERABLE information has accumulated concerning the factor in egg-albumen that neutralizes the biological effectiveness of biotin (Hertz, 1946). This factor has been termed "Avidin" (Eakin *et al.*, 1941) and its presence in both the egg and in the genital tract of several species of birds and amphibia has been demonstrated (Hertz *et al.*, 1942). Moreover, the formation of avidin has been found to be associated with ovarian function in the actively laying hen and avidin production has been experimentally induced in the oviduct of the sexually immature chick by the administration of estrogen followed by progesterone (Fraps, Hertz and Sebrell, 1943; Hertz, Fraps, and Sebrell, 1943).

Trager has described the presence of a fat-soluble factor (FSF) in the plasma of the hen which has biotin-like activity for both the chick and for Lactobacillus casei (Trager, 1947, a). He noted further that this biotin-like factor is elevated in the plasma of the actively laying hen (Trager, 1948).

Accordingly, we have attempted to ascertain the hormonal factors involved in the mobilization of FSF into the blood stream of the sexually immature chick and to examine the relationship of this effect to the concomitant formation of avidin in the oviduct. Our data indicate: (1) that the administration of estrogen to sexually immature chicks induces a marked increase in the total biotin content of the serum, (2) that this increase is attributable to or associated with a rise in both FSF and biotin remaining after FSF separation (true biotin), (3) that although the combined administration of estrogen and progesterone induces marked avidin formation in the

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oviduct, there is no apparent effect of this avidin-formation on the simultaneously elevated level of biotin activity in the blood.

MATERIALS AND METHODS

Newly hatched New Hampshire Red chicks were fed commercial starting mash and tap water ad libitum. They were maintained in electrically heated battery brooders with a fixed period of 12 hours of artificial illumination each day. Beginning on the 8th day of life, diethylstilbestrol and progesterone were administered separately in 0.2 cc. corn oil subcutaneously each day for 8 days in the doses and combinations indicated in Table I. Control animals were completely untreated and accompanied each experimental group. Twenty-four hours after the last injection the chicks were killed by bleeding from the heart and the plasma, oviducts and other tissues prepared for assay.

The plasma was prepared from the heparinized blood by prompt centrifugation. One volume of plasma was mixed with 10 volumes of 3N H_2SO_4 and autoclaved for 1 hour at 15 lbs. pressure. The lipid fraction was separated from this hydrolysate by the method of Trager (Trager, 1947, b). Both the ether soluble and the aqueous fraction were assayed for biotin activity.

For the avidin determination, the magna of the oviducts were carefully dissected and homogenized in normal saline and the assay carried out by the yeast growth test described by Hertz (Hertz, 1943).

Biotin and biotin-like activity was determined by the microbiological method employing L. casei as the test organism (Landy and Dicken, 1942).

RESULTS AND DISCUSSION

The data from a representative experimental series are presented in Table I. It will be seen that the plasma of the untreated chick has

TABLE 1. EFFECT OF STILBESTROL AND STILBESTROL PLUS PROGESTERONE ON BIOTIN LEVELS IN CHICK PLASMA

	Untreated (12 samples)		Stilbestrol (13 samples)‡		Stilbestrol plus progesterone§ (8 samples)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Biotin Activity* (Fat-Soluble Fraction)	3.8	0.79	15	4.4	11	1.8
True Biotin* (Aqueous Fraction)	1.3	0.22	8.3	1.8	10	1.3
Oviduct Weights (mg.)	29.0	6.6	1091.0	192.0	380	93.0
Avidin†	−		−		+	

* Values expressed as milli-micrograms of Biotin per ml. of undiluted plasma.

† (−) signifies less than and (+) more than 0.1 unit of avidin per gram of oviduct magnum (wet wt. basis). A unit of Avidin is the amount required to inhibit completely the yeast growth supported by one microgram of biotin.

‡ Injected subcutaneously with 0.25 mg. stilbestrol in 0.2 cc. corn oil daily for 8 days.

§ Injected subcutaneously with 0.25 mg. stilbestrol in 0.2 cc. corn oil and 1.0 mg. progesterone in 0.2 cc. corn oil daily for 8 days.

a limited biotin activity and that approximately 75% of this activity resides in the fat-soluble fraction. In the estrogen treated birds, and in the estrogen-progesterone treated birds, the total biotin activity in the plasma is elevated about five-fold. In both instances there is a substantial elevation in biotin activity attributable to the fat-soluble fraction (FSF) and to the non-lipoid residue (true biotin). It will be recalled that estrogen alone has been shown to be incapable of inducing avidin secretion whereas a combination of estrogen plus progesterone uniformly induced avidin formation in the young chick (Hertz, Fraps and Sebrell, 1943). Assays of representative oviducts in this series confirmed these earlier observations (Table 1). Thus, the formation of a potent anti-biotin substance in the albumen-secreting portion of the oviduct is not accompanied by an alteration in plasma biotin-activity in either the lipoid or aqueous phase. Moreover, the addition of progesterone to the estrogen had no material effect upon the total rise in plasma biotin-activity.

It should be noted that in accordance with our earlier reports (Hertz, Larsen and Tullner, 1947), the simultaneous administration of progesterone and estrogen substantially inhibits the growth of the genital tract induced by giving estrogen alone (Table 1). It is significant that this inhibition of tissue growth is not accompanied by any depression in plasma biotin-activity, but is accompanied by avidin formation in the oviduct. The role of such metabolites as biotin and avidin in the mechanism of hormone-induced tissue growth remains a challenging enigma. Related observations on the explicit requirement for folic acid in the mediation of hormone-induced tissue growth (Hertz and Tullner, 1949; Kline and Dorfman, 1948) indicate that additional trace factors may play a vital role in this type of tissue deposition.

In addition, the very high biotin content of egg yolk (Kögl and Tönnis, 1936) is not to be considered as simply coincidental with the occurrence of a specific antibiotin substance in the albumen of the egg and in the portion of the genital tract in which the albumen is formed. With the added information that a marked increase in plasma biotin-activity can be effected by estrogenization, one is led to consider that biotin and avidin may have a significant function in the physiology of reproduction.

SUMMARY

Total biotin activity is elevated about five-fold in the blood of the estrogen-treated sexually immature chick. This increase in biotin activity is found in both the fat-soluble fraction (i.e. the FSF of Trager) and in the acid hydrolyzable (or true) biotin. Although simultaneous administration of progesterone with the estrogen induces avidin or antibiotin formation in the oviduct, there is no alteration in the degree of elevation of blood biotin-activity. It is considered

that the hormonal induction of increased levels of biotin activity in the blood and the parallel endocrine stimulation of avidin or antibiotin formation in the female genital tract suggest that these metabolites may play a significant role in the physiology of reproduction.

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BIOASSAY OF HYPOPHYSEAL GROWTH HORMONE: THE TIBIA TEST[1]

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INTRODUCTION

IT HAS been known for some time that pituitary growth extracts stimulate the bony epiphyses of rats and that the increments in the width of the epiphyseal cartilage of hypophysectomized rats as results of growth hormone injections is proportional to the dosage (Li and Evans, 1948). This led Evans *et al.* (1943) to propose a simple bioassay method for estimating growth hormone potency. It was found that the method is highly sensitive and simple as compared to other techniques. In this paper we attempt to standardize the procedure and apply it to the assay of pure and crystalline growth hormone.

PRELIMINARY EXPERIMENTS

Prior to the standardization of the test procedure, certain experiments were done varying the route of administration, the duration of the test period, and the frequency of injections of hormone. In these experiments, both tibiae were removed 24 hours after the last injection, fixed in acetone, and stained immediately with silver nitrate, as described below. The width of the cartilage plate was measured with a calibrated eye piece micrometer and the mean width and the standard error for each group were determined.

Effect of the Mode of Injection. A comparison of the effect of subcutaneous, intraperitoneal and intravenous administration of growth hormone was made in two series of animals. In the first experiment, two groups of female rats, 6 weeks old and 2 weeks post hypophysectomy, received a single daily injection of 0.015 mg. growth hormone for 4 days, subcutaneously and intraperitoneally respectively. There was no significant difference between the mean of the proximal epiphyseal cartilage of the tibia in the two groups (Table 1). In second experiment, 20 male rats, 9–12 weeks old and 3–6 weeks post hypophysectomy were divided into 4 comparable groups. Three groups

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TABLE 1. THE EFFECT OF VARYING THE ROUTE OF ADMINISTRATION OF GROWTH HORMONE ON THE RESPONSE OF THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE TIBIA OF THE HYPOPHYSECTOMIZED RAT

Group	Daily dose	Duration of inj.	Cartilage plate response			
			Subcutaneous	Intraperitoneal	Intravenous	Control†
	micrograms	days	microns	microns	microns	microns
Female rats, 6 wks. old, 2 wks. po.	15	4	235.0±10.3 (7)*	247.0±12.0 (7)	—	159.5±3.0 (34)
Male rats, 9–12 wks. old, 3–6 wks. po.	20	3	135.0±15.6 (5)	143.5± 9.2 (5)	131.8±5.7 (5)	93.8±4.4 (5)

* Mean±standard error; number of animals in parentheses.
† Controls received saline intraperitoneally.

received a single daily injection of 0.020 mg. of growth hormone, for 3 days, subcutaneously, intraperitoneally and intravenously (by tail vein) respectively. The fourth group received saline. Again there was no significant difference between the mean responses of the treated groups (Table 1).

Effect of the Period of Injection. A total of 0.06 mg. of growth hormone was given intraperitoneally in one day, or in divided daily doses for 2, 3 and 4 days. Female rats, 6 weeks old, 2 weeks post hypophysectomy, were used. The results are presented in Table 2. It was noted that this dose of growth hormone administered over 1 or 2 days did not allow a maximum reaction. From the initial studies it appeared that there was no significant difference between the response of the cartilage plate to 0.06 mg. growth hormone when given for either 3 or 4 days. Standard curves were obtained, therefore, with increasing amounts of hormone from 0.02 to 0.2 mg., administered over periods of 3 or 4 days (Table 2). In the 3 day series it was found

TABLE 2. EFFECT OF VARYING THE DURATION AND FREQUENCY OF ADMINISTRATION OF GROWTH HORMONE ON THE RESPONSE OF THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE HYPOPHYSECTOMIZED RAT

Total dose	Cartilage plate response*				
	1 day	2 days	3 days	4 days	5 days
micrograms	microns	microns	microns	microns	microns
10				212.5± 3.7 (23) [197.0± 6.4 (4)]‡	214.5± 3.2 (4)
20			211.8±12.3 (5)	219.0± 6.8 (14)	
40			282.5± 7.1 (4)	265.0± 8.0 (11) [244.8± 6.1 (4)]	281.5± 7.1 (4)
60	175.4±7.2 (5)†	227.2±6.0 (5)	262.0± 4.0 (10)	271.0± 5.6 (10)	
100			296.0±10.9 (4)	319.2± 8.5 (4) [294.5± 7.9 (4)]	299.5± 7.0 (4)
120			261.1± 9.1 (5)	313.9± 4.1 (29)	
200			286.0±15.0 (9)	336.2±11.2 (4) [317.8±14.6 (4)]	320.0±11.5 (5)

* Single intraperitoneal injection was given daily except as noted.
† Mean±standard error; number of animals in parentheses.
‡ Figures in brackets represent the response to the same total dose of growth hormone administered in two daily injections.

that the response "plateaued" at dosage levels of 0.04 to 0.06 mg., and above this dose level did not reach the maximum increase as obtained in the 3 day series. Another group of animals were given increasing doses of growth hormone from 0.01 to 0.2 mg. divided into five equal doses administered intraperitoneally once daily for five days. These results did not differ materially from the 4 day injection series.

Effect of the Frequency of Injection. A group of female rats, 6 weeks old and 2 weeks post hypophysectomy, received increasing amounts of growth hormone from 0.01 to 0.2 mg., intraperitoneally, in divided doses twice daily (at 8 a.m. and 4 p.m.) for 4 days. The mean responses are summarized in Table 2, Col. 5. There was no significant difference in the response between administration of the hormone once daily or twice daily.

From these data it is apparent that a single daily intraperitoneal injection, over a period of 4 days, was a satisfactory plan for the routine bioassay of growth hormone.

THE ASSAY PROCEDURE

Female rats of the Long Evans strain were hypophysectomized at 26–28 days of age and injections of growth hormone were begun after a postoperative period of 12–14 days. The animals were maintained on Stock Diet XIV[2] prior to operation, supplemented by wet Diet I[3] postoperative. A pure growth hormone preparation, prepared as described by Li *et al.* (1944, 1945), was used throughout. For each assay, a small amount of the hormone was carefully weighed and dissolved in 20 cc of saline, to which was added one drop of 1N NaOH. From this stock solution, appropriate dilutions were made in normal saline. The animals received 0.5 cc of the growth hormone solution or of saline (controls), once daily intraperitoneally for 4 days. Twenty-four hours after the last injection, the animals were sacrificed with ether or chloroform, one or both tibias were dissected free from soft tissues and split with a sharp raxor in the mid-sagittal plane. The bone halves were either stained immediately or fixed in 10% neutral formalin. The staining procedure is as described by Evans, Simpson, Marx and Kibrick (1943). Briefly, the bone halves are washed in water one half hour, immersed in acetone at least 1 hour, and washed in water again for ½ hour. They are then placed in freshly prepared 2% silver nitrate for 1 to 2½ minutes, rinsed once in water, and while under water, exposed to a strong light until the calcified portions appear dark brown. They are then immersed in 10% sodium thiosul-

[2] Stock Diet XIV consists of 5% casein, 68.5% wheat, 10% fish meal, 10% alfalfa leaf meal, 1.5% NaCl, and 5% fish oil.

[3] Diet I is slightly modified from McCollum's formula: 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of Vitamin A and 2.5 A.O.A.C. chick units of Vitamin D per gram of diet.

fate for 25 to 30 seconds, and washed in running water ½ hour. They are stored in 80% ethanol, in the dark.

The width of the uncalcified epiphyseal cartilage is measured under the low power of the microscope using a micrometer eyepiece, calibrated with stage micrometer so that the results may be expressed in micra. A minimum of 8 to 10 readings are made across the section and the results are averaged. The cartilage plate increases in width slightly from anterior to posterior. There is, therefore, an error inherent in the measurement of the mean width. This is illustrated in

TABLE 3. THE ANTEROPOSTERIOR MEASUREMENTS OF THE WIDTH OF THE EPIPHYSEAL CARTILAGE FROM THE RIGHT AND LEFT TIBIA OF A CONTROL AND A GROWTH HORMONE TREATED HYPOPHYSECTOMIZED RAT

Width of uncalcified cartilage, in micrometer units*

	Control		Growth hormone treated†	
	Left	Right	Left	Right
	8	7	16	14
	9	7	18	17
	8	7	19	18
	8	9	19	19
	8	8	20	19
	8	7	20	23
	9	9	20	24
	10	10	21	23
	11	10	23	23
	11	10	20	20
Mean and std. error	9.0±0.40	8.4±0.43	19.6±0.58	20.0±1.02

* 1 micrometer unit 17.8 micra.
† Animal received 160 micrograms growth hormone intraperitoneally in 4 days.

Table 3, where the actual micrometer measurements from a right and a left tibia of a control and a growth hormone treated animal are presented. It will be noted that there is a standard error of 0.4 to 1.0 micrometer unit (7.1 to 17.8 micra) within the measurement of a single tibia. This stresses the importance of an adequate number of readings across the entire width of the tibia in the determination of the individual mean. The variations in the width of the cartilage plate are clearly shown in the photographs of stained tibiae from control and growth hormone treated animals (Fig. 1). In a large series there was no significant difference between the left and the right tibia of an animal, so that for routine work, only one tibia need be taken.

STANDARDIZATION OF PURE GROWTH HORMONE

In this study, a total of 205 animals were used. The data were obtained over a period of several months and the results combined. The data are presented in Table 4. Analysis by the method of Least Squares yielded the following equation: $Y = 139.8 + 79.4 \log X$,

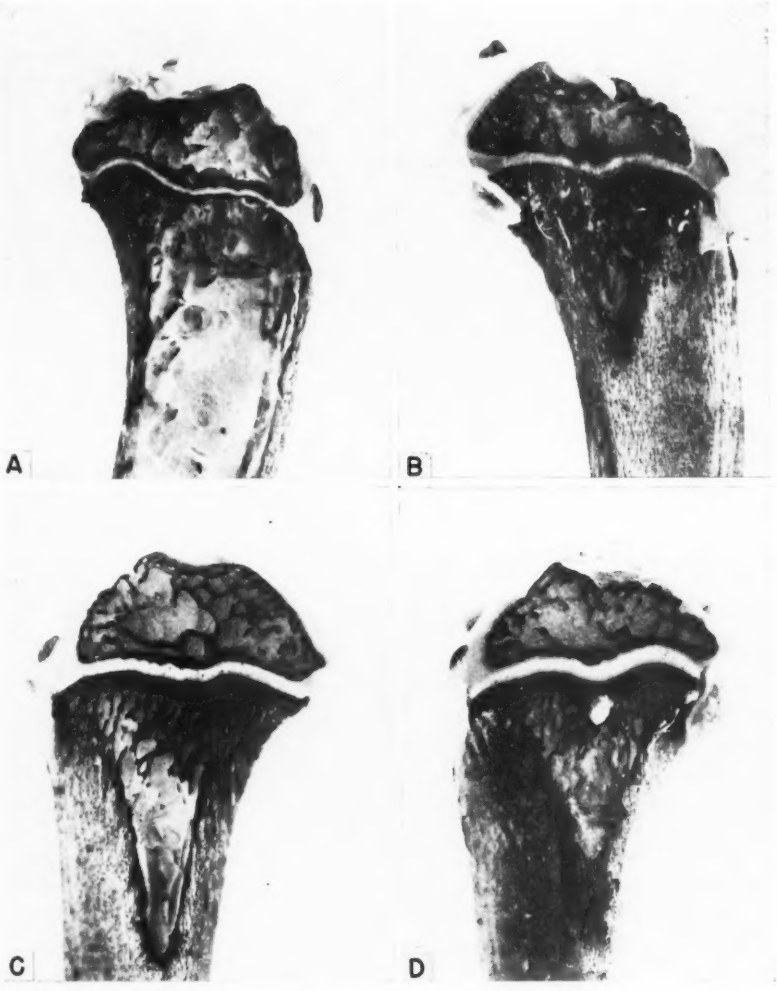

FIG. 1. Effect of Increasing Dose of Growth Hormone on the Proximal Epiphyseal Cartilage of the Tibia of Hypophysectomized Rat in 4 Days. (A) control; (B) 2 micrograms; (C) 16 micrograms; and (D) 120 micrograms.

TABLE 4. THE EFFECT OF PURE GROWTH HORMONE ON THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE HYPOPHYSECTOMIZED RAT

Group number	Total dose	Width uncalcified cartilage	Calculations of "significance"		
			Groups compared	Difference	p†
	micrograms	micra			
1	0	159.5 ± 3.01 (34)*			
2	2	179.4 ± 3.55 (8)	1 vs. 2	+19.9 ± 6.4	< .01
3	5	188.8 ± 8.62 (12)	2 vs. 3	+11.4 ± 10.45	.27
4	10	212.5 ± 3.73 (23)	3 vs. 4	+23.7 ± 7.9	< .01
5	16	237.6 ± 5.80 (10)	4 vs. 5	+25.1 ± 6.67	< .01
6	20	219.0 ± 6.82 (14)	5 vs. 6	−18.0 ± 9.12	.50
7	40	265.0 ± 8.80 (11)	6 vs. 7	+46.0 ± 10.5	< .01
8	60	271.0 ± 5.57 (10)	7 vs. 8	+ 6.0 ± 10.1	.50
9	80	294.2 ± 6.82 (14)	8 vs. 9	+23.2 ± 7.44	< .01
10	120	313.9 ± 4.09 (29)	9 vs. 10	+19.7 ± 7.7	.02
11	160	338.5 ± 7.17 (8)	10 vs. 11	+24.6 ± 9.1	.01
12	240	321.5 ± 8.40 (10)	11 vs. 12	−17.0 ± 10.7	.10
13	320	341.9 ± 8.83 (8)	12 vs. 13	+20.4 ± 11.6	.10
14	800	360.4 ± 4.86 (10)	13 vs. 14	+18.5 ± 9.1	.05
15	1000	364.5 ± 2.97 (4)	14 vs. 15	+ 4.1 ± 7.3	.60

* Mean ± standard error. Number of animals in parentheses; is corrected for small groups.

† From 't' table, Fisher, 1938.

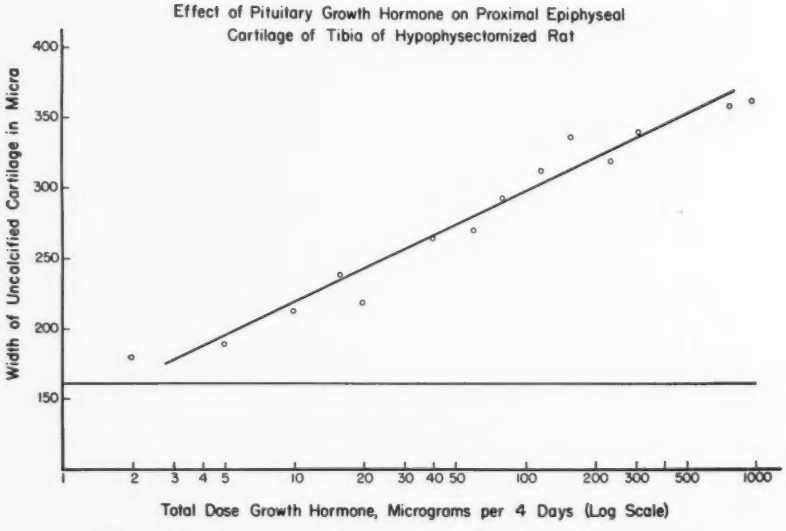

FIG. 2. Effect of Pituitary Growth Hormone on the Width of Proximal Epiphyseal Cartilage of tibia of Hypophysectomized Rat. The Dose-Response Curve.

where Y equals the response of the cartilage plate in micra and X equals the total dose of growth hormone in micrograms. The straight line obtained from this equation, and the means of the responses at each dose level are presented graphically in Figure 2. In order to determine the most satisfactory working range of the test, the data was subjected to the following statistical analysis. At each dose level the mean increment (D) of the cartilage plate response and the standard error of the difference between the given response and the response at the preceding lower dose level (S_D) were calculated for small groups. From the ratio D/S_D a probability value "p" was obtained (Fisher and Yates 1938). These calculations were presented in Table 4, Col. 3, 4, and 5, It will be noted that the responses at the dosage levels of 2 and 5 micrograms were significantly greater than those of the controls, but they were not significantly different from each other. We have arbitrarily considered a total dose of 5 micrograms of pure growth hormone as a minimal effective dose in this series. Between dosage levels of 5 to 120 or 160 micrograms of growth hormone, approximately doubling the dose of hormone produces a significant increment in the response of the cartilage plate. Above a dose level of 160 micrograms, the response levels off somewhat Thus, we consider the dose range of 5 to 120 micrograms of pure growth hormone satisfactory for the tibia test assay. The actual increase in the width of the proximal epiphyseal cartilage of the tibia of the hypophysectomized rat under the influence of increasing amounts of growth hormone is illustrated in Fig. 1. The changes are apparent even under this low magnification.

Calculations were made of the minimal number of animals necessary to obtain a significant response. The mean increment in cartilage width obtained by doubling the dose of growth hormone between 5 and 160 micrograms is 30 micra. Assuming a desired increment in cartilage width of 30 micra, and considering as significant that the ratio of $D/S_D = 3$, then S_D may be ± 10 micra. If we allow a standard deviation of each response to be ± 20.7 micra (the mean of the series), it can be calculated that the minimum number of animals necessary to obtain a "significant" response is 8.5. In practice, groups of 8 to 10 animals should be used for each assay.

DISCUSSION

The effect of hormones other than growth hormone upon the proximal epiphyseal cartilage of the tibia of the hypophysectomized rat was studied in some detail by Marx *et al.* (1944). It was noted that the daily dose of 5 micrograms of thyroxin administered to a group of 31 animals, produced a mean increment in the width of the cartilage plate of 31 micra. Increasing the daily dose of thyroxin to 15 and 30 micrograms did not further increase the response. Thyrotropic hormone, lactogenic hormone, and testosterone at certain dose levels

may increase the width of the cartilage plate from the control level of 155 micra to a range of 175 to 185 micra, but increasing the dose does not increase the response above these levels. There seem to be a number of substances which cause a slight increase in the epiphyseal cartilage plate of the tibia from 160 to about 190 micra, but increments within this range may not be specific effects. Only pituitary growth hormone will stimulate the cartilage above this level and within limits, the response is directly proportional to the log of the dose of the hormone. On the other hand, the response to a given dose of growth hormone may be augmented by certain hormone combinations. The synergistic action of growth hormone plus thyroxin upon the epiphyseal cartilage has been pointed out by Becks *et al.* (1946).

It was demonstrated by Evans *et al.* (1943) with a partially purified growth hormone preparation, that a smaller total amount of hormone was needed in the tibia test assay than in the body weight test assay. The data presented here with growth hormone confirm this observation. Li *et al.* (1945) have shown that a total dose of 90 micrograms of pure growth hormone was necessary to produce a significant response in the 10 day body weight test. It has been noted above that a total dose of 5 micrograms of pure growth hormone will produce a significant response in the standard tibia test assay. The ratio of the minimum amount of hormone required in the two tests is approximately 1 to 10 or 1 to 20.

Gjeddeback (1948) reviewed the accuracy of some of the methods for the bioassay of growth hormone, using the lambda square test (Bliss and Cattell, 1943) as the index of precision. In this analysis $\lambda^2 = S^2/b^2$, where S is the standard deviation of the variation about the curve, and b the slope of the curve as determined by the method of least squares. From our data, $S = \pm 24.6$, and $b = 79.4$, when $\lambda^2 = 0.0945$. This value compares favorably with the accuracy of the methods reviewed in Gjeddeback's report. In addition, it will be noted from Table 4 that within the dosage range of 5 to 120 micrograms of growth hormone, a dose multiple of 2 produces a significant change in the response. It is of interest to compare the present data, obtained with pure growth hormone, with that reported by Marx *et al.* (1944) who employed a less purified preparation. Analysis of the data of Marx *et al.* by the method of least squares reveals the following equation: $Y = 57.1 + 82.5 \log X$, where Y is the width of the epiphyseal cartilage in micra, and X is the total dose of growth hormone in micrograms. The slope of the line, represented by the constant 82.5 is to be compared with the constant 79.4 obtained from the present data. It is of interest to note that by comparison of the tibia test responses, the pure hormone preparation is approximately 9 times as potent as the one used by Marx *et al.*

SUMMARY AND CONCLUSIONS

A standard procedure for the bioassay of growth hormone, which utilizes the increase in the width at the proximal epiphyseal cartilage of tibia of the hypophysectomized female rat has been outlined.

There was found to be no significant difference between intraperitoneal, intravenous and subcutaneous administration of the hormone.

Injection periods of 1, 2, and 3 days were found unsatisfactory, whereas 4 or 5 day injection periods produced a satisfactory response. A single daily injection of hormone produced about the same response as injections of the same total dose given twice daily.

The minimum significant increase in the width of the cartilage plate was considered to be 30 micra.

The most satisfactory working range of the curve lay between approximately 190 and 314 micra, which represented the cartilage response to doses of 5 to 120 micrograms of pure hormone. Within this range, and with groups of 8 to 10 animals, approximately doubling a given dose produced a significant increment in the response.

The slope of the curve obtained with pure growth hormone approached that obtained previously with partially purified growth hormone.

The specificity, sensitivity and accuracy of the procedure are discussed.

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THE CHANGES INDUCED IN IODINE METABOLISM OF THE RAT BY INTERNAL RADIATION OF ITS THYROID WITH I^{131} [1]

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DESPITE the present extensive use of radioactive iodine in the treatment of thyroid disease, little is known of the metabolic changes induced within the thyroid gland by the radiation of the trapped I^{131}. Histological changes have, until now, been the principal guide in determining damage to the gland. In the present study, additional criteria are presented, based on the specialized function of the thyroid gland with respect to iodine metabolism. These are: 1) its capacity to remove iodine from plasma, selectively, 2) its capacity to retain iodine, and 3) its ability to release hormonal iodine into the plasma. An attempt has been made to correlate changes in these three functions with the dose received by the gland following the administration of I^{131}. Similar criteria for estimating radiation effects on the thyroid have recently been employed by Skanse (1948).

EXPERIMENTAL

Male rats of the Long-Evans strain, weighing from 200 to 300 gm., were used throughout. They were fed a stock diet composed of 68.5 per cent wheat, 5 per cent casein, 10 per cent fish meal, 1.5 per cent sodium chloride, 5 per cent fish oil, and 10 per cent alfalfa. This diet, which contained 0.3 micrograms of I^{127} per gram, provided each rat a daily intake of 3 to 5 micrograms of iodine. The animals were kept in a temperature-controlled room and had access to food and water at all times.

I. NORMAL RATS TREATED WITH 24, 300, AND 875 MICROCURIES OF I^{131}

Sixty one rats were injected intraperitoneally with a single dose of I^{131} *to which no carrier iodine had been added.* Each rat received either 24, 300, or 875 microcuries of I^{131}. The rats were sacrificed at intervals of one to eight days thereafter. At the desired interval, the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital

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(20 mg. per rat). Their chests were opened, and 6–8 cc. of blood drawn from the heart directly into a heparinized syringe. Plasma was separated by centrifugation and the protein-bound iodine determined by a method described elsewhere (Taurog and Chaikoff, 1946a, 1948).

The thyroid glands were excised and weighed, and approximately half of a single lobe of each gland was transferred to a fixative for histological study. The remainder of each gland was then weighed and homogenized, with 1 cc. of 10 per cent trichloroacetic acid, in an all-glass apparatus. The homogenate was centrifuged and the precipitate

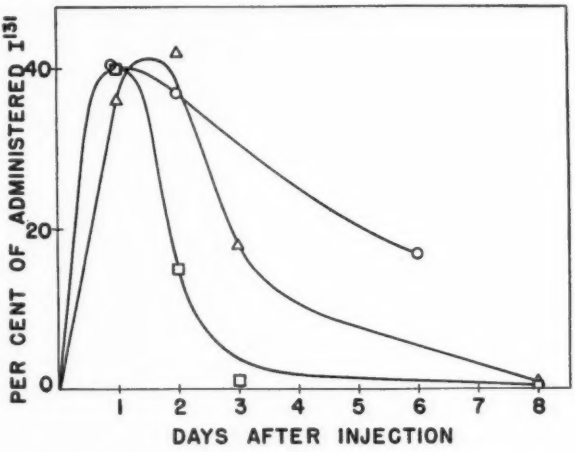

FIG. 1. The uptake of intraperitoneally injected I^{131} by the thyroid glands of rats.
○ 24 microcuries
△ 300 microcuries
□ 875 microcuries

was washed once with 3 cc. of 5 per cent trichloroacetic acid. The combined "supernatants" contained the inorganic iodine fraction of the thyroid. The precipitate (the organic iodine fraction) was dissolved by heating in 1 cc. of 2N NaOH, and diluted to a convenient volume. The chemical iodine content of each fraction was measured as described elsewhere (Taurog and Chaikoff, 1946b). The radioactivity of each fraction was determined by a scale-of-eight Geiger-Müller counter equipped with a thin, mica-window tube.

1. Uptake and Retention of I^{131} by Thyroid Gland in Rats Injected with 24, 300, and 875 Microcuries

The uptake of the injected I^{131} by the glands is shown in fig. 1. The maximum was observed between 24 and 48 hours after injection, and was about 40 per cent of the three dosages.

The rate of loss of the trapped I^{131} from the gland was not the same for the three dosages used. Fig. 1 shows that the greater the radioactivity the of injected dose, the more rapid the depletion of the I^{131} trapped during the first 24–48 hours.

The more rapid loss of I^{131}, observed, when 300 and 875 microcuries, respectively, of I^{131} were injected, indicates that these amounts of radioactivity had injured the gland. Further evidence of damage to the thyroid glands was therefore sought by measuring their contents of total and of organically-bound I^{127}.

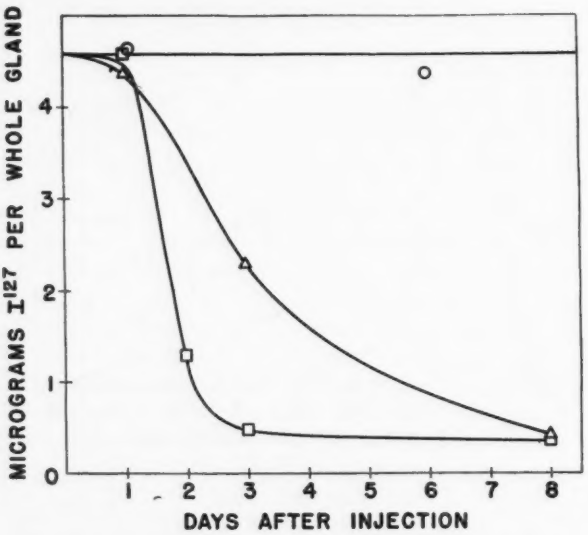

FIG. 2. Loss of chemical iodine (I^{127}) induced in the thyroid gland by injections of I^{131} in rats.
○ 24 microcuries
△ 300 microcuries
□ 875 microcuries

2. Effect of Radiation on the Chemical Iodine (I^{127}) Contained in the Gland

The concentration of organic and inorganic I^{127} in the thyroid glands was measured at five intervals after the injections of the I^{131}—one, two, three, six, and eight days. The results are recorded in fig. 2; each value shown is the average of four observations made on four animals.

No loss of chemical iodine occurred in the glands of rats that had been injected with 24 microcuries of I^{131} for six days. Apparently the dose received by the gland under those conditions does not perceptibly damage the thyroid.

The thyroids of rats injected with either 300 or 875 microcuries did, however, lose considerable amounts of I^{127}. The loss did not occur immediately; none was observed 24 hours after the injection. But by the time two and three days had elapsed, the I^{127} contents of the glands had been reduced to half or less of their initial values, and by the time eight days had elapsed, the I^{127} was practically depleted.

Practically all of this loss in I^{127} involved the organic fraction (Table 1).

TABLE 1. LOSS OF CHEMICAL AND RADIOACTIVE IODINE INDUCED IN THE THYROID GLAND BY INTERNAL RADIATION

Injected dose	Interval after injection	Chemical iodine (I^{127})		Radioactive iodine (I^{131})	
		Organic	Inorganic	Organic	Inorganic
micro-curies	days	micro-grams	micro-grams	per cent of injected dose	per cent of injected dose
24	1	3.7	0.21	39	1.0
24	6	4.1	0.27	16	0.61
300	1	4.1	0.29	35	0.86
300	8	0.55	0.20	0.40	0.23
875	1	4.2	0.32	39	1.0
875	8	0.26	0.10	0.50	0.50

3. The Triphasic Response of Plasma Protein-Bound Iodine to Radiation of the Thyroid Gland

In a recent communication it was shown that, both in its concentration and its rate of formation, protein-bound iodine of plasma responds to experimentally induced changes in thyroid activity in a manner that would be expected of the circulating thyroid hormone (Chaikoff *et al.*, 1947). The injection of thyrotropic hormone increased the concentration of protein-bound iodine of plasma, whereas removal of the thyroids lowered its level. Furthermore, its rate of formation was greatly diminished by thyroidectomy and augmented by injection of the thyrotropic hormone. It is thus evident that protein-bound iodine of plasma is a good index of thyroid activity and for this reason it was used in the present investigation to determine which of the I^{131} doses used had injured the gland.

The changes induced in the concentration of plasma protein-bound iodine by the three doses of I^{131} are shown in fig. 3. No changes occurred in the rats treated with 24 microcuries, an observation that is in accord with the finding of no changes in the I^{127} content of their glands. The values for plasma protein-bound iodine before the injection and on the first, second, and sixth days after the injection of the 24 microcuries were 3.6, 3.6, 3.2, and 3.7 gamma per cent, respectively.

The concentration of plasma protein-bound iodine was reduced as early as 24 hours after the injection of 875 microcuries of I^{131} (fig. 3).

This fall in protein-bound iodine of plasma (referred to here as the initial phase) is the earliest indication so far obtained that the *in situ* irradiation of the thyroid gland had interfered with its metabolic activity.

Plasma protein-bound iodine fell steadily in rats after they had been injected with 300 microcuries. By the time eight days had elapsed, the plasma contained 2.4 micrograms (average) of protein-bound iodine per 100 cc., as compared with an average value of 3.6 gamma per cent before the injections were made.

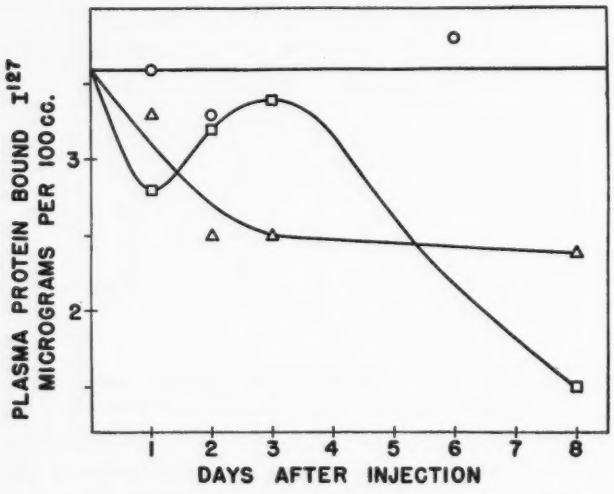

FIG. 3. The response of plasma protein-bound iodine of the rat to injections of I^{131}.
○ 24 microcuries
△ 300 microcuries
□ 875 microcuries

In rats injected with 875 microcuries, an additional change (the second phase) was noted in the level of plasma protein-bound iodine. The initial fall, which undoubtedly was the result of an interference in thyroid activity, was followed by a small, but significant, rise. The time of this rise corresponded with the fall in the concentration of organically bound I^{127} in the gland (shown in fig. 2) and is probably the result of dissolution of thyroid protein induced by the irradiation.

The results of other experiments in which 875 microcuries were injected into rats are shown in fig. 4 and Table 2. It was again observed that an intense radiation of the thyroid gland can induce a pronounced rise in the level of protein-bound iodine, values as high as 7.7 microgram per cent being observed.

The occurrence of this rise in plasma protein-bound iodine at a

time when the gland's iodine is being depleted is also clearly demonstrated in fig. 4.

The third type of response, as shown in fig. 4 and Table 2, consisted of a fall in plasma protein-bound iodine to thyroidectomy levels, i.e., below 1 microgram per cent.

4. Histological Changes

The thyroid tissue was prepared for histological examination as follows. It was fixed in Bouin's fluid, washed for one hour with dis-

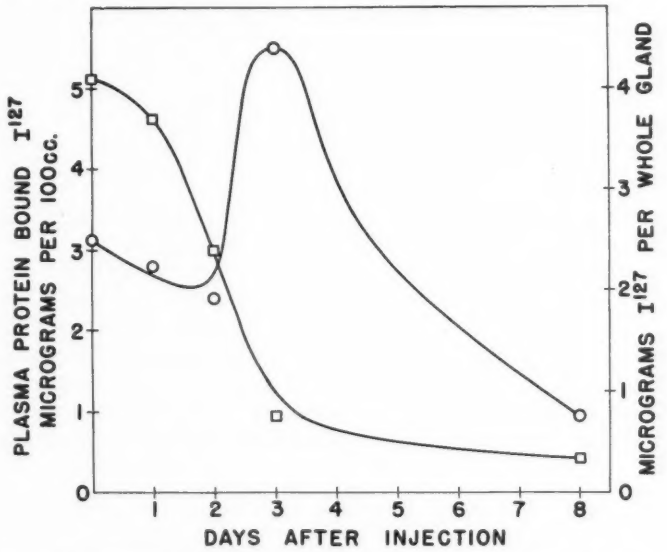

FIG. 4. The relation between the concentration of plasma protein-bound iodine (triphasic response) and the iodine content (I^{127}) of the thyroid gland of rats injected with 875 microcuries of I^{131}.

○ Plasma protein-bound iodine (left abscissa)
□ Whole thyroid I^{127} (right absicissa)

tilled water, and then dehydrated by three successive washings with dioxane. The tissue was embedded in hard paraffin, cut into sections 5 micra thick, stained with Harris' hematolxylin and eosin, and mounted in clarite X. Kidney and thymus were also examined histologically. The thyroids were examined one, two, three, and eight days after the I^{131} injections.

24 Microcurie Injection—No histological changes were detected in glands of rats injected with 24 microcuries.

300 Microcurie Injection—Extensive epithelial damage was found as early as 24 hours in the thyroid of rats that had been injected with 300 microcuries. In 48 hours, edema, leukocytic infiltration, and fibrin reaction were pronounced, but many apparently undamaged

TABLE 2. THE EFFECT OF THE INJECTION OF 875 MICROCURIES OF I^{131} UPON PLASMA PROTEIN-BOUND IODINE

Rat no.	I^{131} injected	Time after injection	Plasma protein-bound iodine
	microcuries	days	microgram per cent
	none (controls)	—	3.1*
1	875	3	5.7
2	875	3	3.9
3	875	3	5.1
4	875	3	7.7
5	875	8	<1.0
6	875	8	<1.0
7	875	8	<1.0
8	875	8	<1.0

* This figure represents the average of six measurements on six rats.

follicles were still present. Eight days after injection, the edema was greatly reduced and many active follicles were observed.

875 Microcuries Injection—24 hours after the injection of 875 microcuries of I^{131}, inflammation, edema, and epithelial degeneration were observed. Extensive destruction, with only a few recognizable follicles, was noted by the time 24 hours had elapsed. By the eighth day, only a few isolated, intact thyroid epithelial cells were present, these being in various stages of degeneration. The inflammatory reaction and edema were still evident, and fibrin deposition was prominent.

II. THE IODINE CONCENTRATING CAPACITY OF IRRADIATED THYROID GLANDS

The following experiment was designed to study the uptake of radioactive iodine by thyroid glands previously irradiated with I^{131}. At three and at ten days after a first injection, these rats were injected a second time with I^{131} (see Table 3). The uptake of this second dose was measured at an early interval—six hours. By the use of suitable controls (those rats that received only the first injection), a correction was made for the activity of the first injection that still remained in the gland; this was usually small as compared with that introduced by the second injection.

The results of this experiment are shown in Table 3. In six hours, the glands of untreated rats had accumulated about 40 per cent of the injected I^{131}. The thyroids of rats that had been injected with 30 microcuries suffered no interference in their iodine concentrating capacity.

Three days after the injection of 300 microcuries of I^{131}, the gland lost most of its capacity to concentrate iodine and no recovery had occurred in 10 days.

III. CALCULATION OF THE AMOUNTS OF RADIATION TO WHICH THE THYROID GLAND IS SUBJECTED WHEN RATS ARE INJECTED WITH 24, 300, AND 875 MICROCURIES OF I^{131}

For purposes of the present investigation, only the effects of the beta rays of I^{131} need be considered, because the half-value thickness of tissue for the gamma rays is many times greater than the linear dimensions of the rat thyroid.

In the case of the small rat thyroid, a geometrical factor must be considered in calculating the dose delivered by beta rays of I^{131} because the maximum range of these particles is comparable to the dimensions of this gland. For any case where ionizations occur beyond

TABLE 3. THE UPTAKE OF RADIOACTIVE IODINE BY THYROID GLANDS PREVIOUSLY SUBJECTED TO INTERNAL RADIATION

Rats	Weights of rats	Weights of thyroid glands	First injection			Second injection	
			Amt. of I^{131}	Duration of action	Estimated dose received by the whole gland	Amt. of I^{131}	Uptake by whole gland in 6 hours
	gm.	mg.	microcuries	days	reps	microcuries	per cent of injected I^{131}
6	256–270	16.5–21.0	30	10	28,000	73	34.5*
6	250–284	18.5–31.5	none	—	—	73	39.5*
4	240–276	20.0–38.5	300	3	150,000	420	3.5†
3	232–310	19.5–30.0	none	—	—	420	32.4‡
3	210–240	19.0–30.0	300	10	175,000	88	3.5‡
3	221–261	27.0–30.0	none	—	—	88	53.2‡

* Average of 6 separate determinations.
† Average of 4 separate determinations.
‡ Average of 3 separate determinations.

the boundaries of a volume of tissue containing radioactive isotopes, a geometrical factor is involved. Marinelli (1948) has dealt with similar factors for gamma ray emitters.

For simplicity, the dose, expressed in roentgen equivalent physical (rep), delivered to the center and to a point near the surface of the rat thyroid is calculated. Geometrical considerations are presented in the addendum.

It is shown that the dose delivered at the center of the gland is

$$D_{\beta^c} = A(1 - e^{-\mu a})$$

and at a point on the surface

$$D_{\beta^s} = A\left[\left(\frac{\mu a - 1}{2\mu a}\right) + \left(\frac{\mu a + 1}{2\mu a} \times e^{-2\mu a}\right)\right]$$

where

μ = absorption coefficient of I^{131} beta particle in cm^{-1}.

a = radius of a thyroid lobe (considered to be spherical) in cm.

The factor A is the accumulated dose in reps originating in the whole thyroid gland. It is given by the following expression (derived below):

$$\frac{N_0(1 - e^{-pt}) \times U \times 2.05 \times 10^5 \times 1.6 \times 10^{-12}}{84 \times w}$$

in which

N_0 = number of radioactive isotopic atoms in the injected dose at zero time.

p = the decay constant, i.e., the fraction of the number of atoms of I^{131} which disintegate per second.

t = time interval in seconds.

U = average per cent uptake of the injected dose by the thyroid gland during time interval, o to t.

w = the weight of the gland in grams.

The terms $(1 - e^{-\mu a})$ and

$$\left[\left(\frac{\mu a - 1}{2\mu a}\right) + \left(\frac{\mu a + 1}{2\mu a} \times e^{-2\mu a}\right)\right]$$

are the geometrical factors for dose at the center and at a point on the surface of the thyroid, respectively.

Let us take the case of a rat injected with 875 microcuries of I^{131}. The total number of disintegrations (given by the expression $N_0(1 - e^{-pt})$) that occurs in such a sample in six days can be obtained as follows:

$$N_o(1 - e^{-pt}) = 875(3.7 \times 10^4)\left(\frac{8 \times 86400}{0.695}\right)[1 - e^{-[(0.695 \times 6 \times 86400/8 \times 86400)]}$$

$$= 13.1 \times 10^{12}.$$

The number of disintegrations that occurs in the gland itself, however, is dependent upon U, the average uptake of the injected I^{131}. The value for U can be obtained from fig. 1 by measuring the area under the uptake curve and dividing by the time interval. It was found to be 12 per cent of the injected dose.

Thus the total number of atoms disintegrating within the gland in six days $= 13.1 \times 10^{12} \times 12/100$ or 15.8×10^{11}.

The emission of each beta particle from I^{131} is accompanied by an average energy output of 0.205 Mev; the energy originating in the whole thyroid gland of the rat will be $15.8 \times 10^{11} \times 0.205 \times 10^6$ electron volts, or 3.2×10^{17} e.v.

The average weight of the rat thyroids used in this experiment

TABLE 4. ESTIMATED RADIATION DOSE AT THE CENTER AND AT A POINT ON THE SURFACE OF THE THYROID GLANDS OF RATS AT INTERVALS AFTER INJECTION OF 24, 300, AND 875 MICROCURIES OF I^{131}

Injected I^{131}	1 Day			2 Days			3 Days			6 Days			8 Days		
	U*	Dose delivered		U*	Dose delivered		U*	Dose delivered		U*	Dose delivered		U*	Dose delivered	
		At the center	At the surface		At the center	At the surface		At the center	At the surface		At the center	At the surface		At the center	At the surface
microcurie		reps	reps		reps	reps		reps	reps		reps	reps		reps	reps
875	30	150,000	63,000	29	280,000	120,000	22	280,000	120,000	12	290,000	120,000	10	290,000	120,000
300	28	46,000	19,000	34	110,000	46,000	32	150,000	63,000	21	170,000	72,000	17	180,000	73,000
24	30	4,000	1,800	33	8,900	3,700	33	13,000	5,300	28	19,000	8,000	—	—	—

* U is the average per cent uptake of I^{131} accumulated by the rat thyroid gland over the interval studied. See text for further explanation.

was 21.1 mg. Therefore, the average energy released per gram of thyroid tissue was

$$\frac{3.2 \times 10^{17}}{21.1} \times 1000 \text{ e.v.}, \quad \text{or} \quad 15.0 \times 10^{18} \text{ e.v.}$$

Since 1 e.v. $= 1.6 \times 10^{-12}$ ergs, and 1 rep $= 84$ ergs per gm., then the above number of electron volts are converted to

$$\frac{(15.0 \times 10^{18})(1.6 \times 10^{-12})}{84} \text{ rep, or } 290{,}000 \text{ reps.}$$

The value for A is shown to be 290,000 reps for the sample calculation.

The geometrical factor for the dose at the center of the gland and at a point near the surface has been shown to be 1.0 and 0.42, respectively (cf. addendum). Therefore, the dose received during six days, at these two points in the thyroid gland of a rat that had been given a single injection of 875 microcuries of I^{131}, was 290,000 reps and 120,000 reps, respectively.

The dose, in roentgen equivalent physical, delivered to these points in the thyroid gland at various time intervals when rats received single injections of 24, 300, and 875 microcuries of I^{131}, was calculated by the above procedure, and the results recorded in Table 4.

DISCUSSION

Table 5 summarizes the various criteria employed in this investigation to assess thyroid function. They are: 1) the histological appearance of the gland, 2) the concentration of plasma protein-bound iodine, 3) the I^{127} content of the gland, 4) the retention of the I^{131} trapped in the gland after the first injection, and 5) the gland's capacity to concentrate a second dose of I^{131}. As judged by all of these criteria, no damage was observed in the thyroid glands of rats ex-

TABLE 5. SUMMARY OF THE CHANGES INDUCED IN THE METABOLIC ACTIVITY OF THE RAT THYROID BY INTERNAL RADIATION WITH I^{131}

Interval after injection when rat was sacrificed	I^{131} injected into rat	Dose at center of the gland	Gland function as judged by the following criteria:				
			Histology	Plasma protein-bound iodine	I^{127} in gland	Retention of first injection of I^{131}	Capacity to concentrate a second dose of I^{131}
days	microcuries	reps					
1	24	4,000	Normal	Normal	Normal	Normal	
1	300	46,000	Abnormal	Normal	Normal	Normal	
1	875	150,000	Abnormal	Abnormal	Normal	Normal	
2	24	8,900	Normal	Normal	Normal	Normal	
2	300	110,000	Abnormal	Abnormal	Abnormal	Normal	
2	875	280,000	Abnormal	Abnormal	Abnormal	Abnormal	
3	300	150,000	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal
3	875	280,000	Abnormal	Abnormal	Abnormal	Abnormal	
6	24	19,000	Normal	Normal	Normal	Normal	
8	30	180,000	Abnormal	Abnormal	Abnormal	Abnormal	
8	875	290,000	Abnormal	Abnormal	Abnormal	Abnormal	
10	30	28,000					Normal
10	300	180,000					Abnormal

amined six days after an injection of 24 microcuries of I^{131}. Nor was damage observed in glands of rats that were sacrificed 10 days after the injection of 30 microcuries of I^{131}. The dose at the center of the thyroid has been estimated for those two doses (see section III and addendum for sample calculation) and shown to be 19,000 reps and 28,000 reps, respectively (Tables 3 and 4). These amounts of radiation are well within the doses employed in this laboratory, during the past 10 years, for studying iodine metabolism in normal, thyroidectomized, and hypophysectomized rats.

The earliest *chemical* indication of radiation damage to the thyroid was provided by the level of plasma protein-bound iodine, a constituent which, as already pointed out, is synthesized mainly in the gland. A decrease in the concentration of plasma protein-bound iodine was observed 24 hours after the injection of 875 microcuries of I^{131}. This decrease, as judged by histological changes in the gland, is the result of an interference in the formation and/or release of thyroxine by the gland, for it has been shown that plasma protein-bound iodine is largely thyroxine (Taurog and Chaikoff, 1948).

The subsequent rise in plasma protein-bound iodine frequently observed on the second or third day after the injection of 875 microcuries of I^{131} probably results from the release of breakdown products of thyroid tissue into the circulation. Such a rise is not surprising in view of the rapid loss of chemical iodine (i.e., I^{127}) from the irradiated glands as shown in figs. 2 and 4. A similar rise in patients treated with large doses of I^{131} has been observed by Riggs (1948).

It should be noted that a rise in plasma protein-bound iodine was not *always* found in the rats that were injected with 875 microcuries of I^{131} and was not observed at any of the intervals investigated in the rats injected with 300 microcuries. Since thyroid necrosis occurred with both of these doses of I^{131}, it seems reasonable to conclude that a rise in plasma protein-bound iodine occurs only when the damage to the gland is so extensive that the resultant rate of release of thyroid iodine into the circulation exceeds the rates of utilization and excretion. Table 4 shows that a dose at the center of the gland of between 50,000 and 150,000 reps per day is required to produce a rise in protein-bound iodine.

A third type of change in protein-bound iodine was observed eight days after the injection of 875 microcuries of I^{131}. The levels of protein-bound iodine at that time were similar to those observed after thyroidectomy (Chaikoff, *et al.*, 1947).

Retention of the injected I^{131} (fig. 1) is also an indicator of thyroid damage, but a less sensitive one than plasma protein-bound iodine (see Table 5). The percentages of the injected I^{131} found in the glands at the end of 24 hours were approximately the same for the three doses of I^{131} used in this study, but the glands subjected to the larger

doses of radiation (300 and 875 microcuries) failed to retain the accumulated I^{131} beyond the first or second day.

By the time 72 hours had elapsed after the injection of 300 microcuries of I^{131}, it became possible to examine another function of the gland, namely, its iodine concentrating capacity as measured by the six-hour uptake of a *second* injection of I^{131}. It was not feasible to make this measurement before 72 hours had elapsed because the second dose of I^{131} would have had to be several millicuries in order to minimize the radioactivity remaining from the first dose. As judged by the uptake of this *second* injection of I^{131}, it would appear that in three days after a rat receives 300 microcuries of I^{131}, its gland loses the capacity to concentrate iodine.

The effects of internal irradiation with I^{131} on several functions of the thyroid of the five-day-old cockerel have recently been studied by Skanse (1948). However, since this worker used a rapidly growing animal whose tissues would be expected to be more sensitive to ionizing radiations than would those of an adult rat, it is difficult to compare his results with those presented here. Interestingly enough, he reported definite inhibition of the growth of the thyroid of the young cockerel 16 days after the injection of only 10 microcuries of I^{131}, during which time he estimated the glands received 13,000 reps. This is a much smaller dosage than that required to affect the adult rat thyroid, as judged by the criteria presented in Table 5.

Skanse also employed two criteria of thyroid function not used in the present study, namely, the response of the glands to thiouracil and to thyrotropic hormone. He observed a decreased response of the chick thyroid to both these agents after exposure of the glands to approximately 60,000 reps.

SUMMARY

100 adult male rats of the Long-Evans strain were injected intraperitoneally with either 24, 30, 300, or 875 microcuries of carrier-free I^{131}, and were sacrificed at one, two, three, six, eight, or 10 days thereafter.

A method for estimating the dose, in reps delivered to the center and to a point near the surface of the small rat thyroid gland, is presented.

The following criteria were used as indicators of metabolic changes induced in the thyroid glands by the internal radiation of the injected I^{131}: 1) the concentration of protein-bound iodine in plasma, 2) the chemical iodine content of the gland, 3) retention of the initial injection of I^{131} by the gland, and 4) the capacity of the irradiated gland to concentrate a second injection of I^{131}. In addition, all glands were examined histologically.

The earliest *metabolic* indicator of thyroid dysfunction was the concentration of protein-bound iodine in plasma.

Following the injection of 875 microcuries of I^{131}, a triphasic re-

sponse in plasma protein-bound iodine was observed. An initial decrease occurred 24 hours after the injection, at which time 150,000 reps had been delivered to the center of the gland. This initial response was followed by a sharp rise in the level of plasma protein-bound iodine. The highest values were found on the third day after the injection of 875 microcuries. The third, or final, response consisted of a reduction in plasma iodine to thyroidectomy levels.

The injection of 300 microcuries of I^{131} decreased the concentration of protein-bound iodine in plasma. With this dose, however, no subsequent rise in plasma iodine was observed; the level fell steadily.

A change in the iodine (I^{127}) content of the thyroid gland was not observed until two days after the injection of either 300 or 875 microcuries of I^{131}. By this time, the center of the gland had received 110,000 and 280,000 reps, respectively. By the eighth day, the gland's iodine was practically depleted.

The percentages of the initially injected I^{131} present in the gland one day after the injection of 300 or 875 microcuries of I^{131} were the same as those observed in rats injected with 24 microcuries. At the end of two days, however, the thyroid glands of rats which had received 300 and 875 microcuries, respectively, failed to retain the trapped iodine to the same extent as did the glands of rats that had received 24 microcuries. This 48-hour interval corresponds to the time when the chemical iodine was being rapidly lost from the strongly irradiated glands.

Rats were treated with 300 microcuries of I^{131}. Three days later the capacity of their thyroid glands to concentrate a second injection of radioactive iodine was determined. The irradiated glands collected about one-tenth the amount of I^{131} found in the glands of the control (untreated) group.

As judged by the metabolic criteria outlined above, thyroid function is not disturbed in the rat up to 10 days after the injection of 24 or 30 microcuries of I^{131}.

ADDENDUM[3]

The problem to be considered is the effect of radioactive iodine (I^{131}), distributed throughout the rat thyroid, upon a point at the center of the gland and upon a point near the surface of the gland.

Consider, then, the effect of I^{131} contained in an elementary volume of tissue upon a point, Q, at a finite distant from this volume. The energy absorbed per unit volume of tissue at point Q, due to active material at point P, is equal to

$$\frac{A\mu e^{-\mu r}}{4\pi r^2}\,dv$$

[3] We are indebted to Dr. W. Siri of the Donner Laboratory and to Dr. S. F Neustadter of the Department of Mathematics for the treatment presented here.

where,

r = distance in cm. from any point P in the elementary volume to the given point Q.

μ = absorption coefficient of I^{131} in cm^{-1}.

A = constant representing the dose delivered to the tissue.

dv = elementary volume.

If, for sake of simplicity, we assume the lobes of the thyroid gland to be spherical in shape and of uniform density as regards the emission and absorption of β-rays, the dose received at the center of the thyroid gland would be

$$D_{\beta^c} = A \int_0^a \int_0^{\pi/2} \int_0^{2\pi} \frac{2\mu e^{-\mu r}}{4\pi r^2} r^2 d\psi \sin\theta d\theta dr$$

or

$$D_{\beta^c} = A(1 - e^{-\mu a})$$

where a = the radius of the thyroid sphere in cm.

For dose at a point on the surface of the sphere

$$D_{\beta^s} = A \int_0^{2a} \int_0^{2\pi} \int_0^{\theta} \frac{\mu e^{-\mu r}}{4\pi r^2} r^2 d\psi \sin\phi d\phi dr, \quad \text{where} \quad \cos\theta = \frac{r}{2a}$$

or

$$D_{\beta^s} = A\left(\frac{\mu a - 1}{2\mu a} + \frac{\mu a + 1}{2\mu a} e^{-2\mu a}\right).$$

The average weight of a single thyroid lobe of rats used in this study was 10.6 mg. The radius of a sphere of such weight can be shown to be 0.135 cm.

An approximation for μ can be made if the density of tissue is assumed to be unity. Thus

$$\mu = \frac{22}{E_{max}{}^{1.33}}$$

where E_{max} is the maximum energy of the beta spectrum. This empirical relation holds only when E_{max} is larger than 0.5 Mev (Siri, 1948).

In the case of beta particles emitted by I^{131}, $E_{max} = 0.60$ Mev[4] and

$$\mu = \frac{22}{0.60^{1.33}} = 43.5 \text{ cm}^{-1}.$$[5]

[4] Metzger and Deutsch (1948) have found that there are more than just one E_{max} of I^{131} as previously supposed. However, for simplicity, only the one value of $E_{max} = 0.60$ Mev has been used in this calculation.

[5] This value for the absorption coefficient of the I^{131} beta particle with an $E_{max} = 0.60$ has been recently verified by an actual measurement of its ionization curve (Siri, personal communication).

The geometrical factors now reduce to
$D_{\beta^c} = 1.0$ (center of the sphere)
$D_{\beta^s} = 0.42$ (point at the surface)

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THE EFFECT OF GROWTH HORMONE ON HEPATIC AND RENAL ACID AND ALKALINE PHOSPHATASES

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CHANGES in the concentration of acid and alkaline phosphatases accompanying growth, in tissues other than bone, have been studied in chick embryos, healing wounds, and tumors (Moog, 1946). It therefore appeared of interest to determine whether or not arresting growth by hypophysectomy or stimulating it by injecting growth hormone alters the concentration of these enzymes in the liver and kidney of rats. Such experiments seemed the more pertinent because the growth hormone characteristically produces storage of nitrogen as protein (Lee and Schaffer, 1934; Young, 1945), and arguments for the implication of phosphatase in protein synthesis have been advanced (Moog, 1946). An increase in hepatic alkaline phosphatase in rats treated with adrenal cortex extracts (Kochakian and Bartlett, 1948) and changes in renal alkaline phosphatase during treatment with steroid hormones (Kochakian and Vail, 1944) also indicate the possibility of such a relationship.

EXPERIMENTAL

The diets, feeding techniques, and animals were those used in preceding papers (Mathies and Gaebler, 1949; Gaebler *et al.*, 1949). The enzyme analyses were carried out according to the procedures outlined by Kochakian (1945), using a substrate pH of 9.1 for the alkaline and 5.4 for the acid phosphatase. Total nitrogen determinations were made on portions of the tissue homogenates by the micro-Kjeldahl procedure, according to Hiller, Plazin, and Van Slyke (1948).

RESULTS

Table 1 summarizes results obtained in the mature male rat. In this experiment only a limited weight gain (17 gm. per rat for the treated group as compared with 4 gm. per rat for the control group) was obtained. This limited response is, however, quite characteristic of the male animal (Evans and Simpson, 1931). Also, no significant change in the concentration of either of the two enzymes determined in liver and kidney occurred. Certainly, it would seem that no pri-

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mary relationship could exist between these enzymes and the mechanism of action of the growth hormone. These results are in accord with those of Kochakian and Stettner (1948), who found no change in these enzymes in castrated mice after treatment with growth hormone.

Different results are obtained, however, when the immature, hypophysectomized female rat is used as the test animal. The data

TABLE 1. THE EFFECT OF MASSIVE DOSES OF GROWTH HORMONE ON PHOSPHATASES IN THE LIVER AND KIDNEYS OF MATURE MALE RATS*

Group	Avg. terminal organ wt. in gm.	Avg. phosphatase units‡					
		Acid			Alkaline		
		Per gm. tissue	Per mg. total N	Per 100 gm. body wt.	Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
				Kidneys			
Treated†	2.79	14.7	0.47	8.2	74.0	2.36	41.5
Control	2.79	13.8	0.43	8.1	64.6	2.02	38.0
				Liver			
Treated†	10.96	17.0	0.42	37.4	1.4	0.035	3.1
Control	11.13	15.7	0.38	36.9	1.4	0.034	3.4

* 8–10 month old Sprague-Dawley rats. Rockland rat diet fed *ad lib.* Fasted last 24 hr. period. Five rats per group.
† Given 10 mg. growth preparation (1740 R.U./gm.)/day for 14 days.
‡ Mg. phenol liberated per hr. under the standard conditions.

in Table 2 indicate that rather marked changes in enzyme concentration occur after hypophysectomy in young rats. A comparison of the normal and hypophysectomized controls shows that a considerable decrease in renal alkaline phosphatase has occurred. The basis of calculation did not effect the over-all change, the decrease being roughly 60–70%. The statistical significance of this difference is indicated by the fact that $P < 0.01$ (Fisher, 1948). At the same time, little difference exists between the acid phosphatase assay values. Any changes that occurred could hardly be considered significant ($P < 0.1$; > 0.05). Upon examining the data obtained on the liver, it appears that the acid phosphatase concentration is slightly lower in the hypophysectomized controls than in the normal controls ($P < 0.01$; $t = 6.44$). However, as these animals were not fasted before sacrifice, to avoid the introduction of another variable, the tissue was probably somewhat diluted with glycogen and lipid. Calculation on the basis of activity per mg. of total nitrogen reduces the difference ($P < 0.01$; $t = 5.07$), while calculation on the basis of activity per 100 gm. body weight inverts the difference, hence it cannot be regarded as very significant ($P < 0.4$; > 0.3; $t = 1.01$). Hepatic alkaline phosphatase in the hypophysectomized controls has undergone a marked increase in concentration of 2.4 – 3 fold, depending upon the manner of calculation. The calculated significance for this difference, based on con-

centration per gm. of tissue, is $P < 0.01$. The effects of hypophysectomy, then, can be summarized by saying that the acid phosphatases of the liver and kidneys remain essentially unchanged, while renal alkaline phosphatase is greatly reduced at the same time that hepatic alkaline phosphatase is greatly increased.

Data given in Table 2 show no significant change in the enzymes of either the kidney or liver of normal animals treated with growth hormone. Such findings are in agreement with the results obtained with mature male rats in the previous experiment. Turning to the

TABLE 2. THE EFFECT OF GROWTH HORMONE TREATMENT ON THE RENAL AND HEPATIC PHOSPHATASES OF IMMATURE, HYPOPHYSECTOMIZED, FEMALE RATS*

Group	Avg. terminal organ wt. in gm.	Avg. phosphatase units					
		Acid			Alkaline		
		Per gm. tissue	Per mg. total N	Per 100 gm. body wt.	Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
				Kidneys			
Hypophysectomized							
Treated	1.10	19.7	0.65	9.7	128	4.20	63.4
Control	0.88	21.8	0.76	10.8	51.2	1.80	25.3
Normal							
Treated	1.24	19.1	0.64	11.2	128	4.30	74.7
Control	1.34	18.7	0.60	13.0	140	4.50	97.4
				Liver			
Hypophysectomized							
Treated	5.39	17.7	0.48	42.8	1.6	0.044	3.9
Control	5.80	14.7	0.43	47.9	2.3	0.067	7.4
Normal							
Treated	4.86	19.1	0.50	43.8	0.88	0.023	2.0
Control	4.62	19.0	0.52	45.5	0.94	0.026	2.3

* Two months old at operation. 5-7 Sprague-Dawley rats per group. Treatment with 1 mg. growth hormone daily per rat (1384 R.U./gm.) for 24 days started 2 weeks after operation. The hypophysectomized controls were fed the diet of Bennett, *et al.* (1948) *ad lib.*, with the remaining groups pair-fed against them. The weight gains of the groups in gms./rat/24 days were: 50, 4, 25, and 5 gms. for the hypophysectomized treated, hypophysectomized control, normal treated, and normal control groups respectively.

effects of growth hormone therapy on the hypophysectomized animals, it is obvious that treatment fully restored the normal complement of renal alkaline phosphatase, calculated on a per mg. of nitrogen basis. Calculation on the basis of activity per 100 gm. body weight reduces the apparent response, due to the low rate of kidney growth as compared with the body weight growth. Hepatic alkaline phosphatase is reduced from its high value in the hypophysectomized animal, although the restoration to normal values is by no means as complete as that obtained with renal alkaline phosphatase.

A similar experiment, of 17 days duration, in which both treated

and control hypophysectomized animals were fed *ad lib.*, while their respective normal control groups were pair-fed against them, gave essentially the same results as in the experiment reported here.

DISCUSSION

When growth is interrupted by hypophysectomy or accelerated by means of growth hormone, alkaline phosphatase concentrations in different organs and tissues do not all change in the same direction. In the tibia, the concentration is low in hypophysectomized animals; it is restored to normal or above by growth hormone, and these changes are readily correlated with arrest and stimulation of skeletal growth (Mathies and Gaebler, 1949). In the kidney the changes are qualitatively the same, but in the liver they are reversed. It follows that each tissue must be analyzed directly, and that changes in alkaline phosphatase concentration of tissue deduced from analysis of serum are not always valid.

It is obviously futile to attempt correlation of changes in hepatic and renal alkaline phosphatase with growth and protein synthesis. Growth is an integral of many reactions. Enzymes catalyze specific reactions, and the functional significance of the reaction which alkaline phosphatase catalyzes differs in different tissues. Presumably, it supplies free phosphate in bone and free glucose in liver and kidney. The decrease in alkaline phosphatase of the kidney and its simultaneous increase in the liver after hypophysectomy might be related to accelerated carbohydrate metabolism in such animals (Bennett, 1936; Russell, 1936; Fisher, Russell, and Cori, 1936). This should increase the demand upon the liver for blood sugar, in the maintenance of which alkaline phosphatase is involved (Cori and Cori, 1938). Whether or not it is compensatory, the rise in liver alkaline phosphatase is in accord with rapid loss of glycogen in fasted hypophysectomized rats (Russell, 1936). Renal alkaline phosphatase is presumably involved in resorption of glucose (Moog, 1946) which should be diminished when oxidation of glucose is accelerated. The decrease of alkaline phosphatase in the kidney could thus reflect diminished need, or it could be part of the general decline in kidney function which White *et al.* (1949) reported in hypophysectomized dogs. However, these investigators found that growth hormone greatly augmented renal clearances in intact dogs, while we find that alkaline phosphatase in the kidney is not increased in intact rats by growth hormone.

The constancy of acid phosphatase concentrations in liver and kidney can be correlated with growth only in the sense that this enzyme behaved like other proteins during arrest or stimulation of growth, and not in any manner indicating a specific relationship. Even in the case of tibia alkaline phosphatase (Mathies and Gaebler, 1949) one can say that observed changes in concentration were

directly related to cessation and stimulation of skeletal growth, rather than to growth hormone, since, in unpublished experiments, no *in vitro* effect of growth hormone on tibia alkaline phosphatase was observed.

SUMMARY

Hypophysectomy of immature, white female rats resulted in an increase in the concentration of hepatic alkaline phosphatase and a decrease in the concentration of renal alkaline phosphatase, while the concentrations of renal and hepatic acid phosphatases remained unchanged. Treatment of these animals with growth hormone partially restored values of hepatic alkaline phosphatase to normal, and completely restored those of renal alkaline phosphatase. The possible relationship of these changes to altered carbohydrate metabolism in the hypophysectomized rat is discussed. The acid phosphatases of both organs remained unchanged in hypophysectomized rats during treatment. Acid and alkaline phosphatases in liver and kidneys were unchanged in the intact rat by growth hormone treatment.

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OXYGEN CONSUMPTION AND PLASMA PROTEIN-BOUND IODINE FOLLOWING ELEMENTAL IODINE INJECTIONS[1]

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IOWA CITY

DVOSKIN (1947) reported findings which indicated thyroxine-like action of subcutaneously injected elemental iodine (I_2), such as speeding up the retarded growth rates of thyroidectomized rats, decreasing adrenal gland hypertrophy following thyroidectomy and diminishing the hypertrophy and hyperplasia of the thyroid in thiouracil-treated rats. It seemed probable that these effects were exerted through formation of thyroxine-like substances, possibly at the very site of injection of the I_2. If so, one would expect to find (1) an elevation in plasma protein-bound iodine (PI) and (2) an increase in metabolic rate as thyroxine or a similar compound was absorbed from the region of production.

These changes actually do result, although the increases in plasma PI are far greater in magnitude than the metabolic effects.

METHODS

The experimental animals used were young adult male and female Sprague-Dawley albino rats, 200 to 350 gms. in weight, the females being consistently lighter than the males. The animals were fed on Purina Laboratory Chow; for the rats on thiouracil, 2 grams of the drug[2] were thoroughly mixed with a kilo of ground food. Thyroidectomies were performed under ether anesthesia, and the animals were not used until two or three weeks later. A careful search was routinely made at autopsy for gross residual or regenerated thyroid tissue and any likely-looking material was examined histologically. All data reported from thyroidectomized rats are from animals meeting this criterion, but it is acknowledged that the possibility exists that other thyroid residues were undetected.

I_2 requires nearly twice as much iodine in the form of sodium or potassium iodide to put it into a water solution. Preliminary experiments revealed that 6.6 mg. NaI (used to dissolve 3.3 mg. I_2) injected per kilogram of body weight per day for 8 days raised the plasma PI of thiouracil-treated rats from

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[2] Thiouracil used in these studies was generously furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, N. Y.

1.63 μg. per cent to 4.75 μg. per cent. This complication was removed by dissolving the I_2 in propylene glycol. The only iodine involved was thus the I_2 itself. As the study was progressing, a note appeared from Dvoskin (1947), pointing out the use of liquid petrolatum as a solvent for the I_2. We continued to use propylene glycol, however, because it was satisfactory as a solvent and could be metabolized by the animals in contrast to mineral oil. Two injection levels were used, 4 mg. and 16 mg. per kilogram per day, each dose in 1 cc. of propylene glycol per kilogram. The desired amount of resublimed I_2 was dissolved with the aid of mild heat and considerable trituration. The solutions were stored in glass-stoppered flasks, great care being taken to avoid any possible contamination of samples of tissues being analyzed for PI or solutions used in the determination.

The injections were made subcutaneously through a fine syringe needle to minimize leaking of the solution. The sites of injection were scattered so that any single location would not be used twice in less than 4 or 5 days. By this means open sores were avoided, although adhesions were often produced.

Oxygen consumption measurements were made with a modified Benedict (1930) multiple-chamber closed-circuit apparatus. The animals were maintained on a special feeding regime (Barker, unpublished) so that they were post-absorptive every afternoon, and repeated determinations could be made without debilitating the animals by repeated fasting.

Protein-bound iodine determinations were run using the procedure described previously (Barker, 1948), modified by the substitution of arsenite for sulfite in the trap of the iodine still (Barker, and Lipner, 1948).

RESULTS AND DISCUSSION

As the most effective presentation of the data, the results of the oxygen consumption studies are shown in graphic form only, as figures 1, 2, and 3. Each curve represents the average of 6 animals treated as indicated with propylene glycol, 4 mg. or 16 mg. I_2 per kilo-

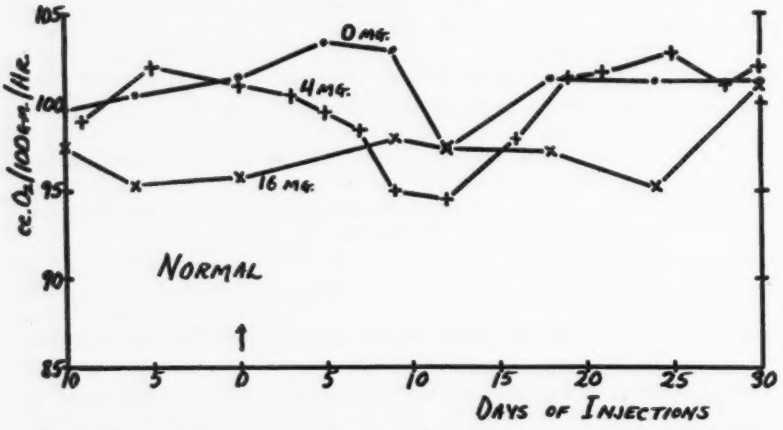

FIG. 1. Oxygen consumption of normal albino rats injected with propylene glycol, 4 or 16 mg. I_2 per kilogram body weight per day in propylene glycol solution.

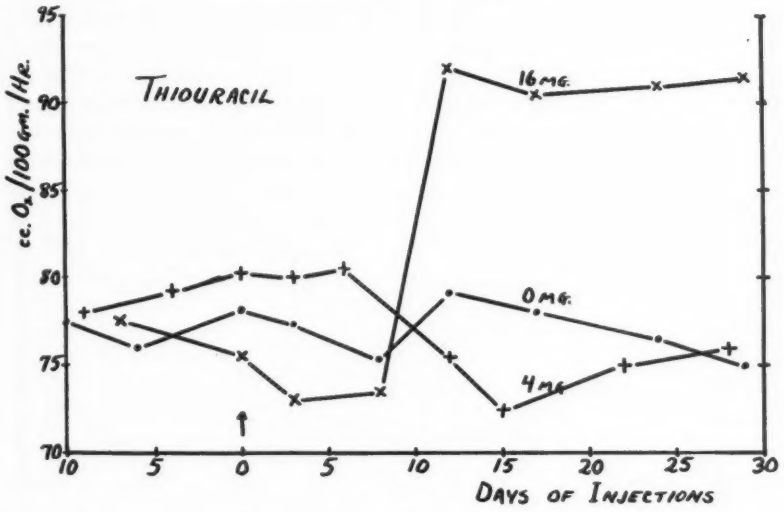

FIG. 2. Oxygen consumption of thiouracil-treated rats injected with propylene glycol, 4 or 16 mg. I_2 per kilogram body weight per day in propylene glycol solution.

gram body weight per day in propylene glycol. Each group is approximately evenly divided between males and females, with no sex differences found in response to the procedures.

Figure 1 reveals no increase in metabolic rate following injection of either 4 or 16 mg. I_2 into normal rats. The only change which seems to follow any sort of pattern is a lowering in the 4 mg. group for the first 10 days, then a return in the next 10 days. The change is small, being of the order of 6 per cent.

The thiouracil-treated animals receiving 4 mg. I_2 per kilogram per day (cf. figure 2) also showed a decrease in oxygen consumption, amounting to about 9 per cent by the fifteenth day. There was only a partial return toward the starting level by the end of the experiment. No ready explanation can be offered for these apparent depressions. In contrast to these fluctuations, a 24 per cent stimulation of metabolism resulted after 12 days on the 16 mg. regime with a latent period of some 8 days. The increase was well maintained for the remaining 17 days of the experimental period.

Examination of the results on the thyroidectomized animals summarized in figure 3 reveals a considerably greater response to the 16 mg. dose, and the first appearance of a metabolic rise from the 4 mg. I_2 per kilogram per day. As can be seen from the graph, the increase resulting from the lower iodine dose was only about 12 per cent, but was consistent. A stimulation of 37 per cent resulted from the 16 mg. injections. Both of these responses were clear by the fifth or sixth

days of treatment and by 10–12 days had reached levels which were sustained to the end of the experiment.

The results of other work, as yet unreported, indicate that the increased oxygen consumption resulting from 4 mg. I_2 per kilogram per day in the thyroidectomized rats is less than that following from the injection of 4 μg. thyroxine iodine per kilogram per day.[3] The elevated

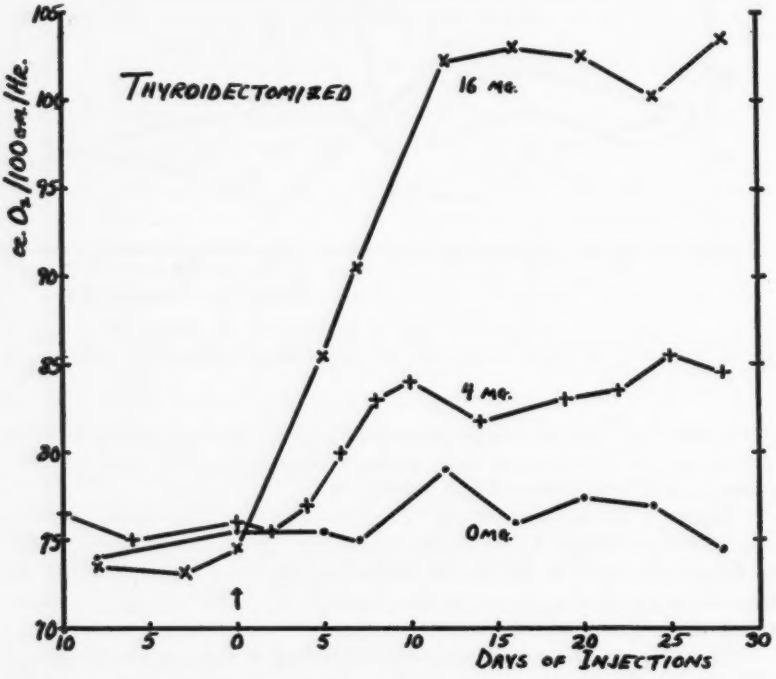

FIG. 3. Oxygen consumption of thyroidectomized rats injected with propylene glycol, 4 or 16 mg. I_2 per kilogram body weight per day in propylene glycol solution.

metabolic level with the 16 mg. dose of I_2 is between those obtained from 8 and from 40 μg. thyroxine iodine per kilo per day. The only comparable information at present available on thiouracil-treated animals is that 4 μg. thyroxine iodine per kilogram per day does not change the metabolic rate within two weeks. A repeated dose of 8 μg. per kilogram per day has no effect on the normal animal. The inference may be drawn from these data that, at least in the thyroidectomized rats, the equivalent of approximately 1 μg. of thyroxine iodine was formed from each 1 mg. of elemental iodine injected. This may be computed as one-tenth of one per cent efficiency, far less than many of the in vitro iodination processes. However, no special pre-

[3] Thyroxine was supplied by Dr. K. W. Thompson of Organon, Inc., Orange, N. J.

cautions to improve the efficiency of thyroxine formation could be taken with the animals, whereas this is possible in vitro.

Terminal blood samples were drawn from the animals used for the metabolism studies and the plasma protein-bound iodine (PI) values are shown in table 1. All rats receiving I_2 injections exhibited greatly elevated PI levels of 70 to 120 μg. per cent with 4 mg. I_2 per kilogram per day and 260 to 340 μg. per cent with 16 mg. I_2. As yet, there is no information concerning the specific nature of the increased plasma PI, whether all thyroxine, which seems unlikely on the basis

TABLE 1. PLASMA PROTEIN-BOUND IODINE IN RATS RECEIVING ELEMENTAL IODINE

	Plasma protein-bound iodine μg. per 100 cc.		
	PG	4 mg. I_2	16 mg. I_2
Controls	5.3	81.9	266
Thiouracil	1.4	72.5	338
Thyroidectomized	1.3	120.0	268

of evidence discussed earlier, or mostly other iodination products, such as monoiodo- and diiodotyrosine. Studies are in progress on this important point.

Although the plasma PI of the thyroidectomized rats receiving 4 mg. I_2 per kilogram per day is higher than the other two groups also on this dose, a similar distinction does not exist at the 16 mg. level, and it is improbable that the greater sensitivity of the thyroidectomized animals can be explained simply on the basis of a greater production of PI. Preliminary data have been reported from this laboratory (Barker and Lipner, 1948) to show that the formation of the PI almost certainly occurs directly at the site of injection of the I_2 probably by a non-enzymatic process similar to the in vitro iodination of proteins in general (cf. Reineke, 1946). In such a case, the presence or absence of the thyroid gland would not be expected to exert any influence. The athyroid animal shows a greater metabolic response to a standard dose of thyroxine than does either normal or thiouracil-treated. The responses found in this work can be interpreted most directly along such lines of differing sensitivity of the different animal preparations to thyroxine formed from the injected elemental iodine.

SUMMARY

Groups of normal, thiouracil-treated and thyroidectomized rats have been injected with propylene glycol alone and 4 or 16 mg. elemental iodine per kilogram per day dissolved in propylene glycol, for 30 days.

Study of the metabolic rates during the period of injections showed no effect in the normals of either levels of I_2. A 24 per cent increase in the thiouracil-treated rats resulted from 16 mg. I_2, but

no change from the 4 mg. dosage in this group. The lower amount of I_2 produced a 12 per cent, and the greater a 37 per cent elevation in oxygen consumption of the thyroidectomized animals.

Considerable elevations in plasma protein-bound iodine of all animals were produced by both levels of I_2, the 4 mg. dose causing rises to 70–120 μg. per cent and 16 mg. to 260–340 μg. per cent. The increases were approximately the same in all three types of animals, indicating that the thyroid gland was not involved in the production of the protein-bound iodine.

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A STUDY OF THE METABOLISM OF RAT TESTIS IN VITRO[1]

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INTRODUCTION

DURING the course of a study of the effect of various gonadotrophins on rat testis it seemed pertinent to correlate previously described changes in chemical morphology (Tepperman and Tepperman, 1947) with the metabolism of the gland *in vitro*. A survey of the literature revealed that Schuler (1944) and Meier and Schuler (1941) had made careful studies of the metabolism of surviving testis. The present study is an outgrowth of experiments that were done for the purpose of confirming an observation made by these authors.

In 1944, Schuler reported that the Q_{O_2} of normal adult testis and that of testicular tissue obtained from weanling rats is about the same in the presence of added glucose. However, when glucose was omitted from the medium, the Q_{O_2} of surviving gland obtained from adult rats was very low, whereas that of the weanling rat's gonad was slightly higher without added substrate than it was in the presence of 0.2% glucose. Schuler attributed this difference in metabolism to the fact that the weanling rat's testis is comparatively "undifferentiated." In this report the hypothesis is developed that a low oxygen uptake in the absence of added substrate is a characteristic of the germinal component of the testis. Moreover, it is suggested that, as the cells of the seminiferous tubules grow to constitute a larger and larger percentage of the gland mass, their distinctive metabolism obscures the metabolic pattern of the non-germinal elements in the gland.

MATERIALS AND METHODS

Albino Farms rats were used throughout this study.

Transplantation of the testis was done with clean, but not aseptic, technique through a mid-line abdominal incision in rats anesthetized with pentobarbital sodium. Stilbestrol pellet implantation was performed according to

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the technique described by Nalbandov and Baum (1948). The 15 mg. stilbestrol pellets were kindly supplied by Mr. Fred Houghton of the Ciba Company. Q_{O_2} was measured over a period of one hour on decapsulated, teased testis tissue in phosphate buffer at a pH of 7.4. Oxygen was the gas phase, the bath temperature was 37.7°C and the shaking speed was 120 per minute. The representative dry weight method was used in the calculation of Q_{O_2} and dry weight included total solids. Glucose was added to the medium where indicated in a concentration of 0.2%. Measurements of respiratory quotient (R.Q.) were made according to the method described by Umbreit *et al.* (1945). The lactic acid method used was that of Barker and Summerson (1941). The chorionic gonadotrophin employed was Follutein, Squibb, and it was supplied through the courtesy of Dr. R. Bates. For the testosterone propionate in sesame oil we are indebted to Mr. Houghton of the Ciba Company.

Means and their standard errors are presented throughout. A difference between means which exceeds three times the standard error of the difference is regarded as significant.

EXPERIMENTAL DESIGN AND RESULTS

1. *Effect of age on metabolism of testis* in vitro.

Results of this experiment are shown graphically in Figure 1. It will be noted that the figures for Q_{O_2} given for 26 gram rats and 300 gram rats constitute good confirmation of the report of Schuler (1944). The intermediate values for 50, 104, 150 gram rats clearly show that, while there is no significant difference in the oxygen uptake

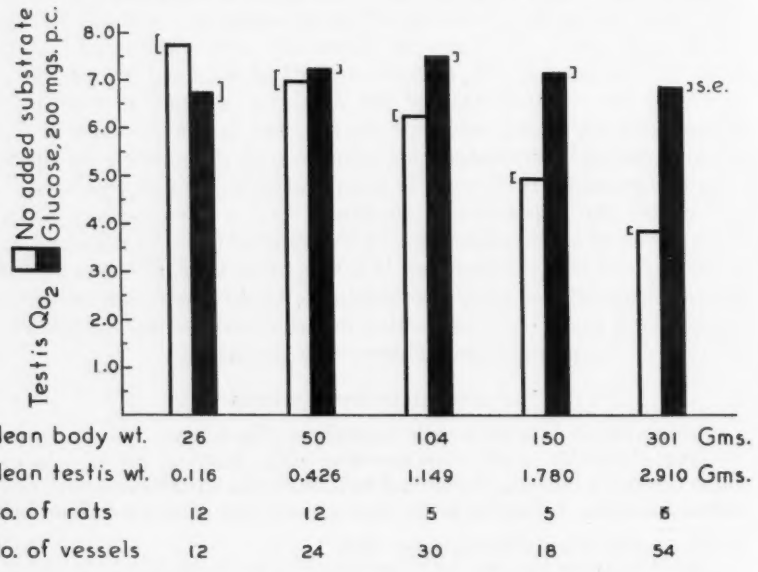

FIG. 1. Effect of age on Metabolism of Testis *in vitro*.

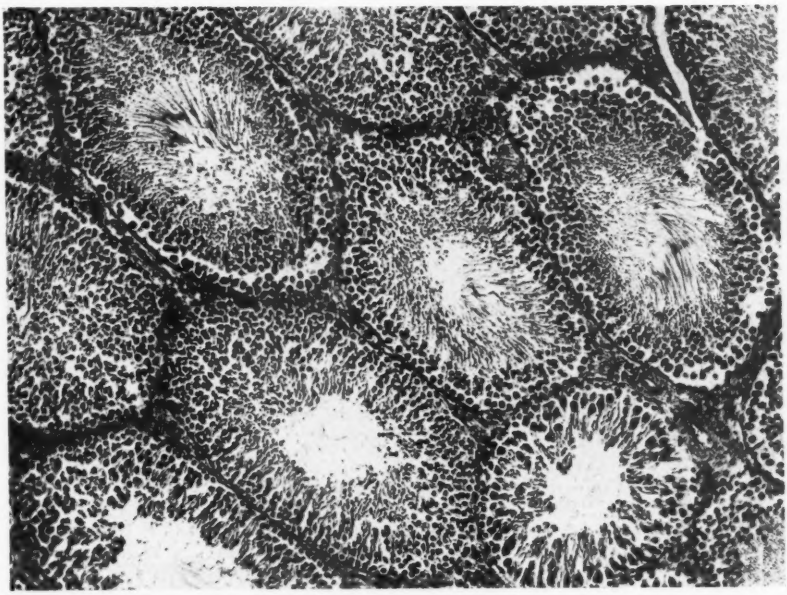

FIG. 2A. Scrotal testis, Experiment 2. X 115.

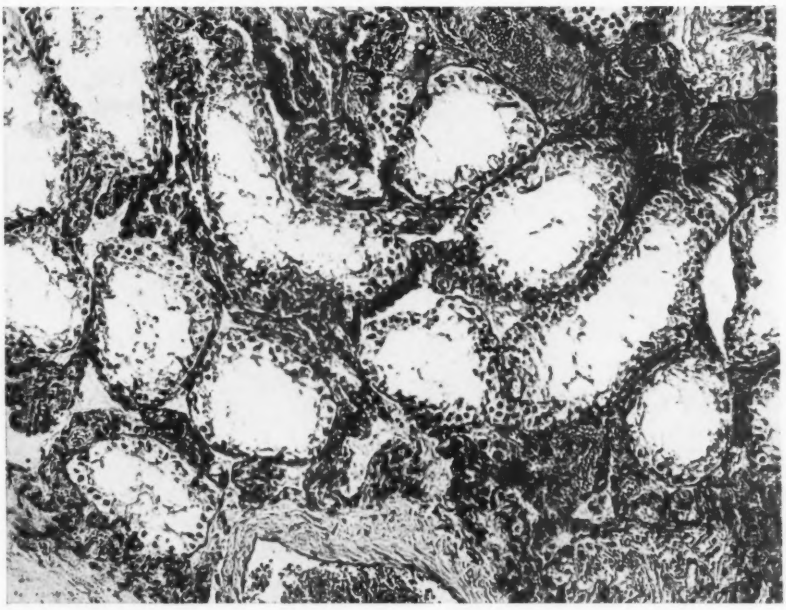

FIG. 2B. Transplanted testis, Experiment 2. X 115.

of the gland in the presence of added glucose, there is a progressive fall in Q_{O_2} with advancing age in the absence of added substrate. Since this fall was associated with a progressive rise in the mean diameter of the seminiferous tubules it became pertinent to discover whether or not the increasing mass of germinal tissue was masking the metabolism of the non-germinal moiety of the gland.

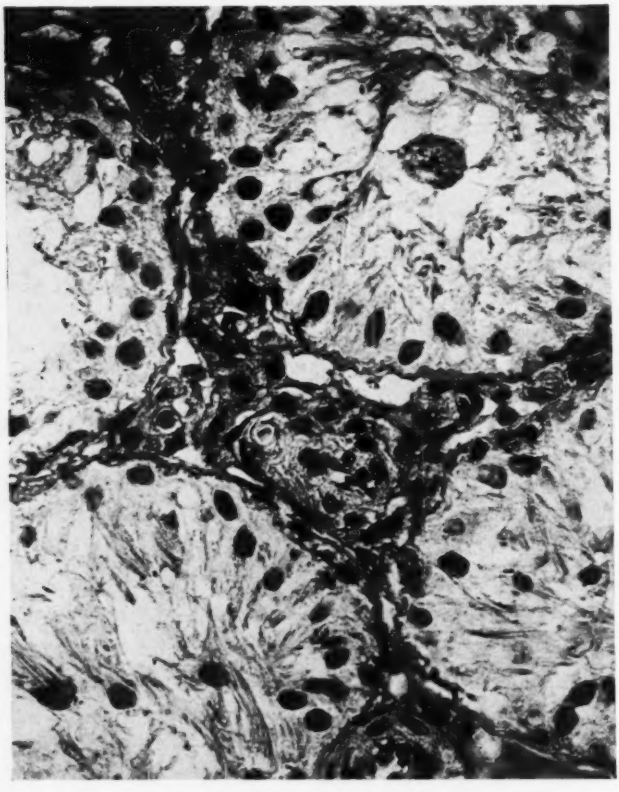

FIG. 2C. Transplanted testis, Experiment 2. X 550.

2. *Effect of atrophy of seminiferous tubules on the metabolism of testis* in vitro.

Atrophy of the seminiferous tubules was produced deliberately by the technique of experimental cryptorchidism. Rats that weighed between 250 and 300 grams were anesthetized and the capsule of one testis was transfixed with a single silk suture and attached to the posterolateral abdominal wall of the homologous side. Between 23 and 28 days after operation the rats were killed, the testes weighed,

and tissue was taken for oxygen consumption measurements. At the time of sacrifice the rats weighed 287 ± 5.6 grams. The transplanted testis weighed 155 ± 6 mgs. per 100 grams of rat, as compared with the scrotal testis which weighed 445 ± 10 mgs. per 100 grams of rat. The scrotal testis did not differ significantly in weight from those of normal control animals, nor was there any readily detectable differ-

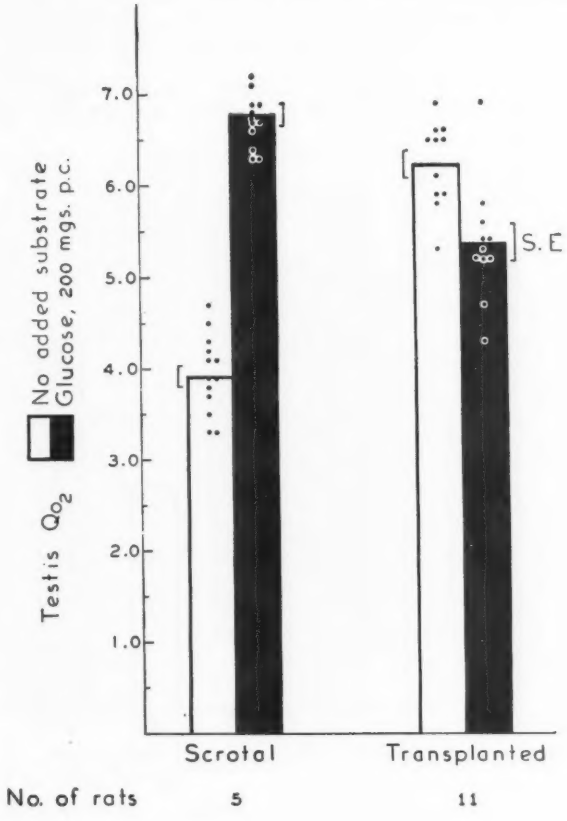

FIG. 3. Effect of atrophy of seminiferous tubules on the metabolism of testis *in vitro*.

ence in histology between the scrotal testes and those taken from unoperated animals of the same age.

The histological appearance of these glands is shown in Figures 2A, 2B and 2C. The extreme atrophy of the seminiferous tubules of the transplanted gland is well shown in Figure 2B. Figure 2C is included to demonstrate the persistence of Sertoli cells in the atrophic

tubules. Sudan stains of similar preparations showed a very marked increase in Sudanophilic lipid in the cryptorchid glands.

The metabolic pattern of the scrotal and transplanted glands is shown in Figure 3. It is noteworthy that there is no difference between the pattern observed in the scrotal gland and that seen in unoperated animals of similar size. However, the transplanted testis demonstrates a highly significant increase in oxygen consumption in the absence of

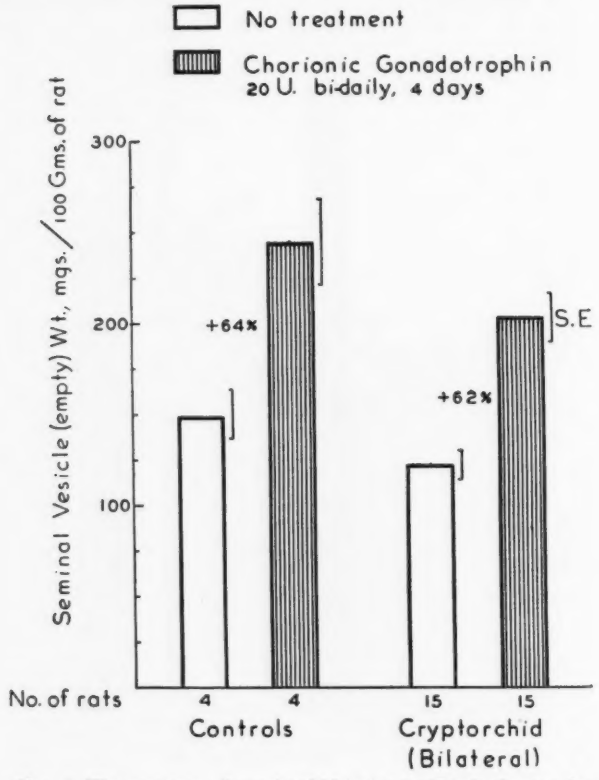

FIG. 4. The response of cryptorchid testes to Chorionic gonadotrophin administration.

added substrate as compared with the scrotal gland. Moreover, this tissue shows a smaller oxygen consumption in the presence of glucose than it does in its absence.

To determine whether or not the transplanted testis is functionally differentiated, a number of bilaterally cryptorchid animals were given chorionic gonadotrophin for four days and the resulting enhancement of androgen output indirectly determined by the seminal vesicle weight technique. The results of this experiment are given in Figure 4. The enhancement of androgen production in cryptorchid animals

given chorionic gonadotrophin was not significantly different from that seen in a representative group of controls. (See Nelson, 1934)

3. *Respiratory quotient and aerobic glycolysis of normal adult, infantile, and cryptorchid testes.*

Since the cryptorchid gland appeared to be less dependent upon the presence of added glucose in the medium than did the scrotal gland, the possibility arose that these tissues might well be meeting their energy requirements by oxidizing different substrates. Therefore,

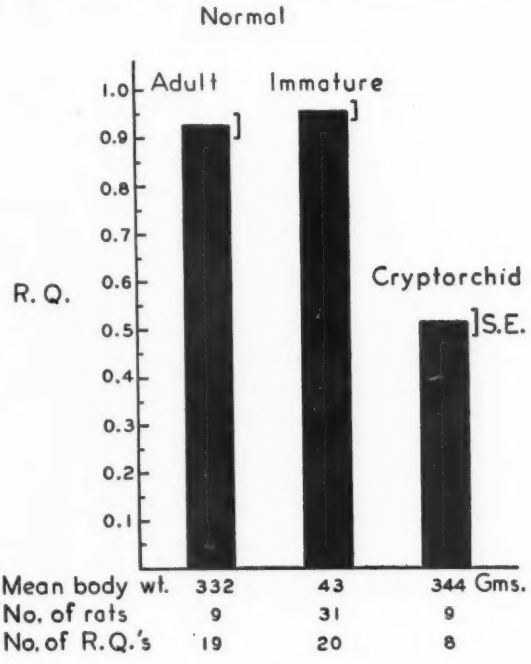

FIG. 5. The respiratory quotient (R. Q.) of normal adult, immature and cryptorchid testes.

R.Q. measurements were made on adult, immature and cryptorchid glands. The results of this experiment are given in Figure 5. There is a highly significant difference between the glucose R.Q. of the cryptorchid gland and that of normal adult gland, but there is no significant difference between the R.Q.'s of immature testis and the adult one.

It is of some methodologic interest that it was technically feasible to make R.Q. determinations on adult and immature testis tissue with little difficulty. However, in the beginning, the R.Q. values for the cryptorchid tissue were extremely variable and many of them were

palpably absurd. For example, in some individual determinations, the initial CO_2 vessel appeared to contain more CO_2 than was produced in the 60 minute CO_2 vessel. This difficulty persisted until a correlation was made in the experimenters' minds between the degree of absurdity of the results and the number of visibly calcified tubules that were seen in the tissue samples. Accordingly, samples were taken from which visibly calcified tubules were rigorously excluded and the respiratory metabolism of these tissues was compared with that of

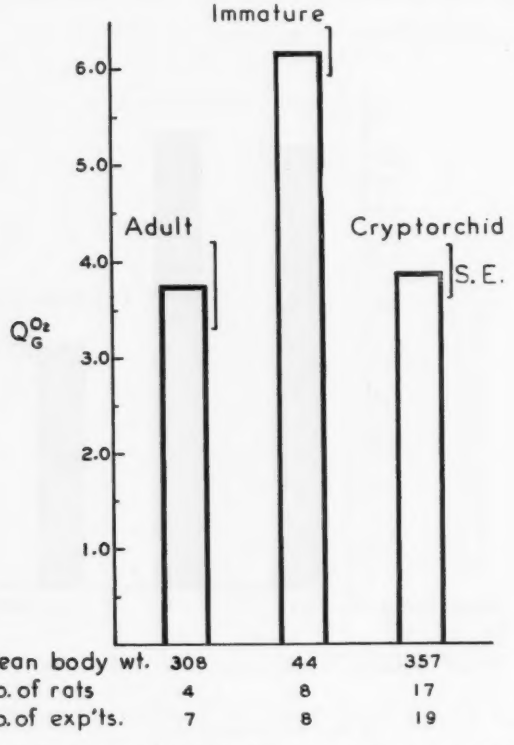

FIG. 6. The aerobic glycolysis of normal adult, immature and cryptorchid testes.

samples which were deliberately contaminated with calcified tubules. The former showed readily reproducible R.Q.'s whereas the latter gave impossibly varied ones. The values reported here for the cryptorchid testis, then, are ones which were obtained from tissue in which there was no visible calcification. It is suggested that the original error was probably based upon the release of CO_2 from $CaCO_3$ contained in calcified material when the acid was tipped in.

Since Reiss and others (1933) had shown that the aerobic glycoly-

sis of immature rat testis was much higher than that of the adult tissue, an experiment was performed in which this parameter was measured in adult, immature and cryptorchid glands. The results of experiments are shown in Figure 6. In confirmation of previous work (Reiss, 1933), it will be seen that the immature glands have a comparatively high aerobic glycolysis whereas there is no significant difference between the lactic acid production of the cryptorchid gland and that of the adult gland.

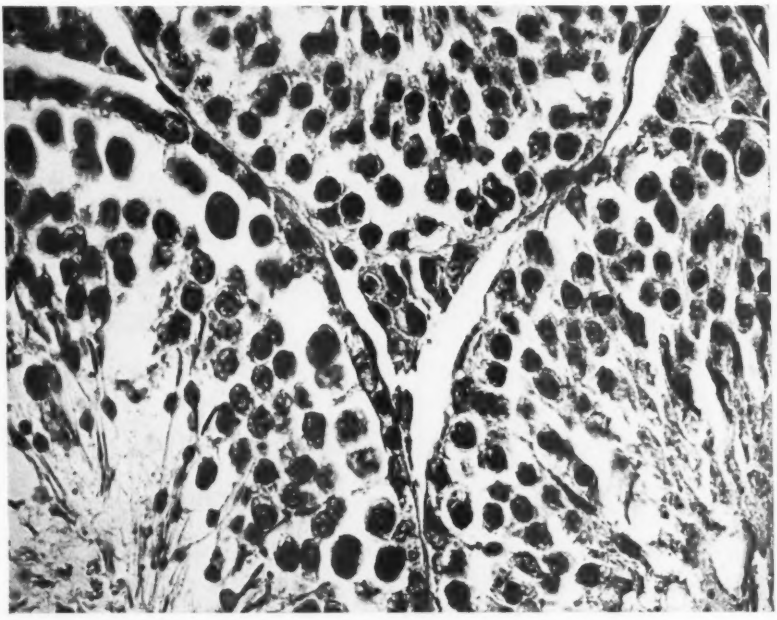

FIG. 7A. Normal testis, 150 gm. rat. X 550.

4. *Effects of androgens and estrogens on testis metabolism.*

A group of animals weighing approximately 150 grams each were treated for 7 days with 1 mg. of testosterone propionate per day. At the end of that time they were sacrificed and respiratory metabolism of their testis tissue was compared with that of suitable controls. Histologically there was a striking decrease in the interstitial cell mass which is well shown in Figures 7A and 7B. Whereas there was no significant change in the glucose Q_{O_2}, the testosterone treated animals showed a small but statistically significant decrease in the Q_{O_2} without added substrate (Table 1). The effect of testosterone was greatly magnified when a single experiment was performed on bilaterally cryptorchid animals. In this experiment, as Table 1 clearly shows,

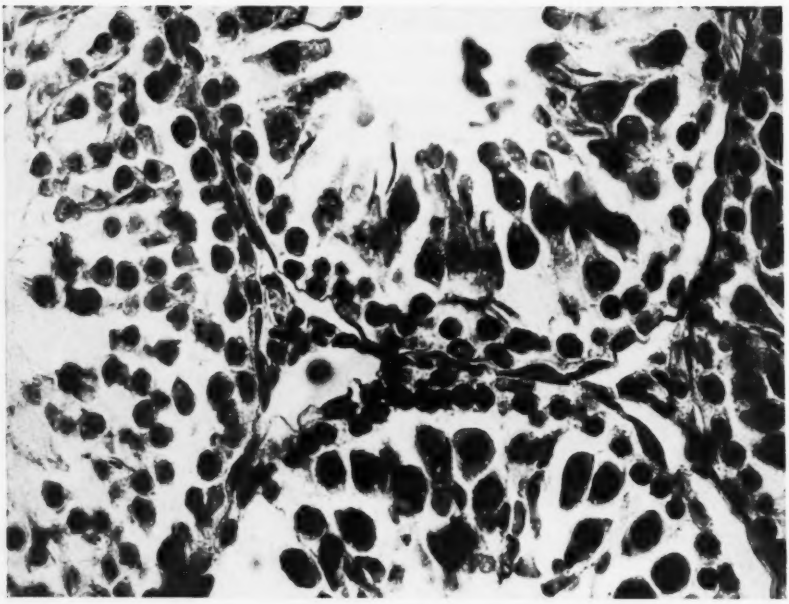

FIG. 7B. Testis of testosterone-treated 150 Gm. rat (1 mg. testosterone propionate in sesame oil for 7 days). X 550.

there was a striking fall in both the glucose Q_{O_2} and the Q_{O_2} without added substrate.

Following the report of Nalbandov and Baum (1948), who described a technique of performing a "medical hypophysectomy" with respect to pituitary gonadotrophins, an experiment was performed on the testes of rats in which stillbestrol pellets had been implanted 24 days before. The effects of this procedure are described

TABLE 1. EFFECT OF TESTOSTERONE ON THE RESPIRATORY METABOLISM OF INTACT AND TRANSPLANTED TESTES

Treatment	Number of rats	Mean body weight	Number of vessels	Testis Q_{O_2}	
				No added substrate	0.2% glucose
Controls	5	150 ± 3.7	9	5.0 ±01.2	7.2 ±0.10
Testosterone Propionate in Oil (7 mgs. in 7 days)	4	138 ± 2.6	8	4.2 ±0.14	7.0 ±0.14
Cryptorchid	17	287 ± 5.57	11	6.2 ±0.14	5.4 ±0.20
Cryptorchid Testosterone Propionate (7 mgs. in 7 days)	7	343 ±25.9	12	4.6 ±0.25	3.7 ±0.27

TABLE 2. THE OXYGEN UPTAKE OF TESTES OBTAINED FROM STILBESTROL-PELLET INPLANTED RATS

Treatment	Number of rats	Mean body weight	Testis weight mgs./100 gm. rat	Number of vessels	Testis Q_{O_2}	
					No added substrate	0.2% glucose
Controls	5	158±5.5	1229±86.5	6	3.7±0.21	6.6±0.07
15 mg. pellet stilbestrol implanted 30 days	12	115±2.6	129± 5.5	6	5.3±0.22	4.6±0.31

quantitatively in Table 2. The marked lowering of Q_{O_2}. and the apparent reversal of the effect of adding glucose to the medium are well shown. The absolute values for glucose Q_{O_2} in the pellet-implanted animals, however, are not as low as are those given by Schuler for hypophysectomized animals.

DISCUSSION

The experimental results reported herein are consistent with the hypothesis that the germinal epithelium of the seminiferous tubules and the remaining cells of the testis have qualitatively different types of metabolism. In the adult gland the germinal elements are so numerous that their metabolic performance is scarcely "diluted" by that of the other cells. When the seminiferous tubules become atrophic, a different metabolic pattern is brought into relief. This pattern represents the activity of a constellation of cells that includes the endocrine portion of the gland, the Sertoli cells, connective tissue cells and blood vessel cells. It is obvious that the androgen-producing elements of the interstitial tissue constitute a larger portion of this constellation than they do of normal, adult testis. Therefore, a preparation such as the one described here may prove to be of particular value in the elucidation of the mechanisms involved in androgen production, and, especially, in the role of gonadotrophins in augmenting the rate of synthesis and release of male hormone.

Although the differences between the metabolism of the transplanted and scrotal glands are quite striking, the figures reported above probably represent only a fraction of the true differences. Since the cryptorchid glands contained a much larger amount of Sudanophilic material than did the scrotal ones, calculation of oxygen consumption on the basis of fat free dry weight would magnify the differences between the tissues. Furthermore, as Fig. 2B shows, the cryptorchid gland appears to contain rather large amounts of fibrillar material that would not be expected to contribute significantly to the total oxygen consumption of the tissue. Therefore, if nucleic acid phosphorus or some similar reference factor had been used instead of dry weight, the differences between the oxygen consumption of the two tissues would have been even more greatly magnified.

The contribution of the Sertoli cells to the total metabolism of the cryptorchid gland is a question of some interest, since, in some prep-

arations, cell counts revealed an approximate ratio of Sertoli cells to interstitial cells of 1:1. If, as Huggins and Moulder (1945) have suggested, Sertoli cells are a source of estrogen, their metabolism might be expected to resemble that of androgen-producing cells rather than that of germinal epithelial cells.

Although there is a certain similarity between the metabolism of cryptorchid and immature testis, Figs. 5 and 6 make it clear that these tissues can be differentiated on the basis of criteria other than the Q_{O_2} with and without added glucose. Immature testis resembles the adult gland in its glucose R.Q., whereas it differs from both adult and cryptorchid tissue in aerobic glycolysis.

The experiment described in Table 1 indicates that a procedure designed to produce atrophy of the androgen-producing cells of the testis may have only a slight effect on the metabolism of whole testis, whereas the same maneuver in bilaterally cryptorchid rats results in a more marked inhibition of oxygen consumption. Conversely, preliminary experiments have indicated that increases in oxygen consumption produced by gonadotrophin injection can be greatly magnified if measurements are made on cryptorchid glands.

Finally, it is noteworthy that glands of stilbestrol pellet-implanted rats have a Q_{O_2} of 5.3 ± 0.22 without added substrate and 4.6 ± 0.31 with added glucose. The corresponding figures for hypophysectomized rats given by Schuler (1941) are approximately 3.9 and 3.6. These differences may be related to the fact that, in the estrogenized animal, there is no evidence of a deficiency of thyrotrophic or adrenotrophic hormones (Nalbandov and Baum, 1948). The comparatively marked depression of testicular metabolism in the hypophysectomized rat may be an expression of deprivation of other trophic hormones as well as of gonadotrophins.

SUMMARY

The Q_{O_2} of rat testis does not change significantly over a wide age range if glucose is present in the medium. However, in the absence of added substrate, there is a progressive fall in Q_{O_2} with advancing age (Fig. 1).

Atrophy of the seminiferous tubules was produced by the technique of abdominal transplantation. The Q_{O_2} of the remaining tissue (which consisted principally of interstitial cells and Sertoli cells) was measured and found to be significantly higher than that of normal adult tissue in the absence of added substrate. The addition of glucose to the medium produced a lowering of the oxygen uptake (Fig. 3).

The glucose R.Q. of cryptorchid testis is 0.5 as compared with a value of 0.93 obtained with normal adult tissue (Fig. 5). The aerobic glycolysis of cryptorchid testis does not differ from that of normal adult glands (Fig. 6).

Androgen produces a lowering of the no-substrate Q_{O_2} in intact glands. The androgen effect is magnified in cryptorchid glands (Table 1).

The testes of stilbestrol-implanted rats have a higher Q_{O_2} than that previously reported for the glands of hypophysectomized rats (Table 2).

The implications of some of these findings are discussed.

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THE TEMPORARY NATURE OF THE INHIBITORY ACTION OF EXCESS IODIDE ON ORGANIC IODINE SYNTHESIS IN THE NORMAL THYROID[1]

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In 1944, Morton *et al.* observed that when 300 mg. of sheep thyroid slices were incubated in 3 cc. of a bicarbonate-Ringer medium to which various amounts of I^{127} as inorganic iodide had been added, inhibition of thyroxine and diiodotryosine formation occurred when the added I^{127} exceeded 20 gamma. The resemblance of this *in vitro* effect to the action of excess iodide in Graves' disease in man led us to investigate this phenomenon in the normal intact thyroid (Wolff and Chaikoff, 1948a). In addition to confirming the inhibitory action of excess iodide upon thyroxine synthesis in intact thyroids, the investigation showed that such inhibition by a *single injection* of iodide was related to the level of plasma iodine (Wolff and Chaikoff, 1948b). So long as the concentration of plasma iodine exceeded 20–35 gamma per cent, organic binding of iodine failed to occur in the gland, and only when the concentration fell below this critical range did the gland resume its function of depositing iodine in an organic form. These observations in rats have been confirmed recently by Stanley (1948).

Evidence of an inhibitory action of excess iodide on thyroid function has also been obtained by a different approach. Purves and Griesbach (1946) have shown in rats that excess iodide (one mg. per day) potentiates the antithyroid action of a weak goitrogen. The administration of thiourea alone resulted in a slight degranulation of the acidophils of the anterior pituitary. But when large amounts of iodide were given along with the thiourea, the anterior pituitary presented a picture typical of complete thyroidectomy, i.e., complete degranulation of the acidophils. Mackenzie (1947) also has observed that the antithyroid effect of another weak goitrogen, sulfaguanidine, is augmented in rats fed a diet containing 500 gamma of iodine per gram.

It is well known that in the thyrotoxic patient, administration of excess iodide results in a return of the hyperplastic gland to a more

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[3] Atomic Energy Commission Fellow.

normal state and in an increase in both total and thyroxine iodine of the gland (Gutman *et al.*, 1932). But in the normal rat, excess iodide, as judged by its ability to depress thyroxine formation in the thyroid, *in vitro* (Morton *et al.*, 1944) as well as *in vivo* (Wolff and Chaikoff, 1948b), appeared, rather, to resemble the action of antithyroid substances such as thiourea or its congeners. This resemblance between the effects of excess iodide and the antithyroid substances raised the question: does the maintenance of plasma iodide concentrations, well above 35 gamma per cent, for long periods induce histological changes in the thyroid and pituitary glands characteristic of a deficiency in circulating thyroid hormone? Although several attempts have already been made to study the effects of the administration of large amounts of iodide (Gray and Loeb, 1928; Weil, 1936; Hall, 1946), no reports have yet appeared in which the concentration of plasma iodide is shown to have been maintained at such high levels *without interruption* for as long as one month. Insurance against any interruption whatsoever is necessary because it was found that organic binding of iodide in the thyroid gland (i.e., hormone formation) is resumed when the plasma iodide falls below a critical level in the neighborhood of 20–35 gamma per cent.

A. THE EFFECTS OF INJECTIONS OF 500, 1,000 AND 2,000 GAMMA OF I^{127}, THREE TIMES DAILY FOR 1–4 WEEKS, UPON THE STRUCTURE OF THYROID AND PITUITARY GLANDS

Twenty-seven female rats of the Long-Evans strain, weighing from 166 to 232 grams, were injected intraperitoneally every eight hours for periods of one to four weeks with either 500, 1000, or 2,000 gamma of I^{127} as KI in 0.9 per cent saline (Table 1). All rats were sacrificed 10 hours after the last injection in order to insure adequate testing of blood iodine levels at a time which was two hours beyond the interval between successive injections. They were anesthetized with sodium pentobarbital. Blood was then removed from the heart.

TABLE 1. CONCENTRATIONS OF PLASMA TOTAL IODINE FOUND IN RATS INJECTED INTRAPERITONEALLY WITH IODIDE THREE TIMES DAILY

Amounts of iodide injected every 8 hours	Duration of injections	Plasma total iodine 10 hours after last injection
gamma	days	gamma per cent
500	1	156 –205
500	2	137 –175
500	3	165 –394
500	5	125 –269
500	14	555
500	21	225– 425
1000	14	540– 580
1000	21	265 –375
2000	14	675–1060
2000	21	535– 735

Total blood iodine was determined on two cc. samples of plasma, according to the method of Taurog and Chaikoff (1946).

After exsanguination, the thyroids were rapidly excised, weighed, and fixed in Bouin's. They were embedded in paraffin and prepared in multiple sections five micra thick. These were stained with hematoxylin and eosin. Liver, kidney, and heart were similarly treated.

Immediately after excision of the thyroids, the pituitary glands were removed, fixed in Zenker-Formol (9:1), embedded in paraffin, and prepared in sections 2–3 micra thick. They were stained by the method of Martins (1933) modified according to Griesbach (personal communication). The percentages of acidophils, basophils, and chromophobes were determined by a method similar to those employed Griesbach and Purves (1943) and by Floderus (1944). At least 2,000 cells were counted in each gland.

Table 1 lists the total plasma iodine values of animals killed at 1–5 days, and at two and three weeks after the start of the iodine injections. It will be seen that a minimum plasma iodine value of about 150 gamma per cent is established as early as 24 hours (i.e., after three injections of 500 gamma of iodine as KI), and is maintained at levels far in excess of that previously found necessary to prevent organic binding in the thyroid gland following single injections of 500 gamma of iodine.

Thyroid Histology (*Figs. 1 and 2*).—No evidence of hypersecretion of thyrotropic hormone was observed, i.e., epithelial hypertrophy or hyperplasia, or decrease in follicular colloid. On the contrary, reduction of follicular cell height and an increase in follicular colloid was a consistent finding in all animals that had received the iodide injections for one or more weeks.

Pituitary Cytology (*Figs. 3 and 4*).—Cytological examination of the anterior pituitary glands from these animals was undertaken as a more sensitive means of detecting a goitrogenic effect, if any, of a prolonged high plasma iodide level.

The sensitive response of this gland to very slight thyroxine deficiency has been clearly demonstrated in a series of studies by Griesbach and coworkers (1945, 1949) in thyroxine deficient rats. A decrease of 0.1–0.2 gamma in the amount of injected thyroxine necessary to maintain the anterior pituitary in a normal state can be detected by a significant increase in the percentage of the basophils. A more severe thyroxine deficiency (induced either by subtotal thyroidectomy or by the administration of a weak goitrogen) results in the appearance of large hyalinized basophils (thyroidectomy or signet ring cells) and some degranulation of the acidophils. Complete degranulation of the acidophils occurs only after total thyroidectomy or after prolonged treatment with a highly active goitrogen such as propylthiouracil. (fig. 4).

In Table 2, the anterior pituitary cell counts of the iodide-injected rats are compared with results obtained from the following types of

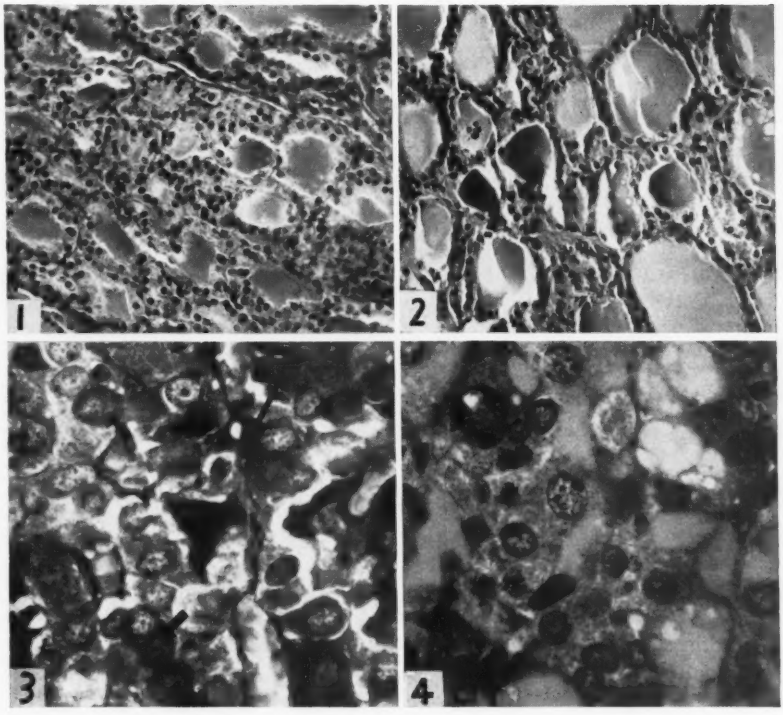

FIG. 1. Thyroid from control rat, maintained on diet containing 0.3 gamma iodide per gram. Note columnar epithelium and large vesicular nuclei. Hematoxylin and eosin; x275.

FIG. 2. Thyroid from rat injected with 2000 gamma iodide every eight hours for three weeks. Note flattened epithelium and smaller deeper staining nuclei. Hematoxylin and eosin; x275.

FIG. 3. Anterior pituitary from animal injected with 1000 gamma iodide every eight hours for three weeks. Arrows in upper portion of picture point to granulated acidophils which appear gray. Arrow at lower left points to a normal basophil. This does not differ from control pituitaries. Martins; x750.

FIG. 4. Anterior pituitary from rat maintained on diet of 0.2 per cent prophythiouracil for one month. The basophils contain large hyaline vacuoles (thyroidectomy cells) and constitute 20 per cent of the total number of cells. All other cells are chromophobes. Martins; x750.

rats: (1) controls, i.e., rats fed the same diet but not injected with iodide; (2) rats totally thyroidectomized; and (3) rats fed for one month a diet containing 0.2 per cent propylthiouracil.

The percentages of the various cell types found in the injected rats were well within the range found for control animals. When the anterior pituitaries of rats exhibiting thyroxine deficiency are compared with those of rats injected repeatedly with large amounts of iodide for periods up to four weeks, it becomes clear that the latter suffer from no deficiency in circulating thyroid hormone.

TABLE 2. DIFFERENTIAL CELL COUNTS OF ANTERIOR PITUITARIES OF RATS

Treatment	Anterior pituitary cell counts*			Remarks
	Acidophils†	Basophils	Chromophobes	
	per cent of total	per cent of total	per cent of total	
Control (untreated)	38–43	5–9	50–57	Cells of normal appearance.
500, 1000, 2000 gamma I^{127} thrice daily for 2 weeks	33–38	6–10	54–56	Cells of normal appearance.
500, 1000 2000 gamma I^{127} thrice daily for 3 weeks	35–41	6–10	48–57	Cells of normal appearance. See fig. 3.
500, 1000, 2000 gamma I^{127} thrice daily for 4 weeks	36–39	5–9	52–58	Cells of normal appearance.
One month after total thyroidectomy	0.7–1.1	21–29	70–78	Complete acidophilic degranulation. Majority of basophils of thyroidectomy type.
0.2 per cent propylthiouracil diet for one month	1.0–1.8	18–28	70–80	Complete acidophilic degranulation. Majority of basophils of thyroidectomy type. See fig. 4.

* At least 2000 cells were counted per gland.
† Degranulated acidophils were included in total per cent chromophobes.

B. FOR HOW LONG CAN THE THYROID GLAND BE INHIBITED WHEN A HIGH LEVEL OF PLASMA IODINE IS MAINTAINED?

It was shown in a previous report that the injection of a *single* dose of 500 gamma of iodide in the normal rat inhibited the formation of organic iodine for about 17 hours (Wolff and Chaikoff, 1948a). When the excretion of the iodide was prevented by nephrectomy, this effect of a *single* injection was prolonged to 32 hours (Wolff and Chaikoff, 1948c). But our failure to observe histological signs of thyroid deficiency in the preceding experiment led us to question the permanence of the inhibitory effect of excess iodide upon thyroxine formation in the gland. The following experiments were therefore designed to determine how long the normal rat thyroid can be inhibited by excess iodide.

A stock solution of KI, containing 500 gamma of I^{127} per cc. and an initial radioactivity of about 20 microcuries of I^{131} per cc., was prepared. One cc. of this *same* solution (i.e., 500 gamma I^{127}) was injected intraperitoneally into normal rats at eight-hour intervals for periods up to 111 hours. The levels of plasma total iodine[4] established by this

[4] Only a small fraction of this iodine is protein bound.

procedure (Table 3 and 4) are so high that dilution by the amount of iodine contributed by diet and breakdown of thyroxine is negligible. As a result, the specific activity of plasma remains the *same* as that of the injected dose throughout the experiment. Because of this, it was possible to calculate the amount of new organic iodine in the thyroid (Wolff and Chaikoff, 1948a).

Experiment 1—The rats used in this experiment weighed 150–222 gm. and, since weaning, had been fed a low iodine diet containing 0.3–0.4 gamma of iodine per gram. They had access *ad libitum* to this diet throughout the period of observation. The animals were sacrificed at 9, 18, 26, 50, 74, and 111 hours after the *first* injection and from 7 to 10 hours after the *last* injection. Blood was obtained from the heart, and the iodine content of plasma determined, as already described. The thyroids were excised and weighed. The organic and inorganic iodine fractions of the thyroids were separated, by means of trichloro-

TABLE 3. THE DURATION OF THE INHIBITORY ACTION OF EXCESS IODIDE IN THE THYROID GLAND

EXPERIMENT 1

(The rats were injected every 8 hours with 500 gamma of I^{127}-labeled I^{131})

Time after first injection	Plasma total iodine*	New† I^{127} in thyroid as:					
		Inorganic		Organic		Total	
hours		gamma	mg. per cent	gamma	mg. per cent	gamma	mg. per cent
9	68	2.9	11.2	0.18	0.69	3.1	11.9
	113	1.2	7.2	0.14	0.87	1.3	8.1
	100	4.0	13.8	0.11	0.38	4.1	14.2
	115	0.98	5.1	0.27	1.4	1.3	6.5
18	138	2.7	10.0	0.31	1.1	3.0	11.1
	183	1.9	11.9	0.42	2.6	2.3	14.5
	185	2.4	12.0	0.38	1.9	2.8	13.9
	140	2.5	11.6	0.30	1.4	2.8	13.0
26	145	2.2	11.4	0.38	2.0	2.5	13.4
	83	0.92	4.6	0.29	1.4	1.2	6.0
	105	2.7	10.4	1.1	4.2	3.8	14.6
	88	3.9	18.1	1.0	6.3	4.9	24.4
50	170	2.3	10.0	2.9	12.6	5.2	22.6
	170	0.89	4.4	5.1	25.5	6.0	29.9
	345	3.2	16.6	1.1	5.8	4.3	22.4
	153	1.7	6.7	2.0	8.0	3.7	14.7
74	252	3.0	15.0	3.4	17.0	6.4	32.0
	302	3.3	13.2	2.6	10.4	5.9	23.6
	178	1.3	7.6	4.6	27.0	5.9	34.6
	240	1.4	6.5	5.8	27.6	7.2	34.1
111	172	1.6	5.6	10.7	38.2	12.3	43.8
	190	1.6	6.7	4.6	20.0	6.2	26.7
	78	0.65	3.8	7.0	41.1	7.7	44.9
	300	0.83	4.0	11.4	54.2	12.2	58.2

* Only a small fraction of this iodine is protein-bound.

† Refers to I^{127} as calculated from the radioactivity recovered in the gland.

acetic acid, as described in an earlier report.[5] The results of this experiment are recorded in Table 3.

It was shown that when the gland escaped from the inhibitory effects of a single injection of excess iodide, the amount of *new* I^{127} organically bound in the thyroid gland was always well in excess of one gamma (see fig. 6 of Wolff and Chaikoff, 1948a). During inhibition, on the other hand the amounts of *new* I^{127} so bound were below this amount. Hence for the present study, we have considered the gland to have escaped from inhibition when the total amount of *new* I^{127} bound organically exceeded one gamma.

In experiment 1 (Table 3), inhibition occurred during the earlier intervals, after the establishment of a high plasma iodine level. In two of the rats, more than one gamma of *new* organic iodine had accumulated in the thyroids 26 hours after the start of the injections. By the time 50 hours had elapsed, the gland showed a renewed capacity to bind iodine organically—*this occurred while the plasma iodine was still high.* At later intervals, more and more of the *new* iodine was organic.

Experiment 2—The rats weighed 210–272 gm. and had been raised on the same low-iodine diet as that used for experiment 1. They were injected every eight hours with 500 gamma of I^{131} labeled iodide, prepared as described in experiment 1, above. All rats were sacrificed two hours after the last injection. For this reason, plasma iodine values, as well as the inorganic iodine content of the thyroid glands, are higher than those observed in experiment 1. These results are shown in Table 4.

Up to 26 hours, the amounts of the injected I^{127} organically bound in the thyroid did not exceed one gamma, the value arbitrarily set as the escape level. At 34 hours, the thyroids of two of the rats still showed inhibition whereas in the other three rats examined at this interval, about 1.3 gamma of *new* I^{127} were organically bound.

Inhibition was still observed in two of the four rats examined at 42 hours and in one of the five rats examined at 50 hours. But 58 hours

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[5] It has already been shown (Wolff and Chaikoff, 1948a) that no inorganic iodide appears in the *insoluble* fraction. The nature of the trichloroacetic acid-*soluble* fraction was tested here by a method similar to that described by Taurog *et al.* (1947). Rats were injected with 500 gamma of I^{127} containing I^{131} and were killed five or 22 hours thereafter. Glands of three rats were pooled and ground with one cc. of cold 10 per cent trichloroacetic acid. The resulting precipitate was washed twice with 2.5 cc. of 5 per cent trichloroacetic acid and discarded.

To the combined supernantants (the trichloroacetic acid-soluble fraction) were added 0.1 cc. of a 2N KI solution, as carrier, and 0.5 cc. of 0.1 N KIO_3. The I_2 so formed was then extracted three times with equal volumes of CCl_4. The combined CCl_4-extracts were then re-extracted three times with dilute (0.1 N) sodium thiosulfate. The radioactivity of the trichloroacetic acid-residue, the CCl_4-fraction, and the thiosulfate fraction was measured. Ninety-five per cent of the iodine originally present in the trichloroacetic acid-soluble fraction was recovered in the thiosulfate solution. This finding indicates that the iodine in this fraction must have been either in the form of I^- or I_2. *This was the case in the early intervals while the excess iodide exerted its inhibitory action as well as later when escape from inhibition had occurred in the gland.*

TABLE 4. THE DURATION OF THE INHIBITORY ACTION OF EXCESS IODIDE IN THE THYROID GLAND
EXPERIMENT 2
(The rats were injected every 8 hours with 500 gamma of I^{131}-labeled I^{127})

Hours after first injection	Plasma total iodine*	New† I^{127} in thyroid as:					
		Inorganic		Organic		Total	
hours	gamma per cent	gamma	mg. per cent	gamma	mg. per cent	gamma	mg. per cent
10	440	9.0	45.0	0.42	2.1	9.4	47.1
10	275	8.5	35.4	0.24	1.0	8.7	44.1
10	260	9.0	36.0	0.28	1.1	9.3	37.1
10	240	7.5	31.2	0.14	0.58	7.6	31.8
18	436	11.0	52.3	0.49	2.3	11.5	54.6
18	366	8.5	42.5	0.34	1.7	8.8	44.2
18	410	8.0	36.4	0.80	3.6	8.8	40.0
18	320	9.5	36.5	0.37	1.4	9.9	37.9
18	470	10.0	40.0	0.26	1.0	10.3	41.0
26	340	9.0	40.8	0.70	3.2	9.7	44.0
26	240	5.0	26.3	1.0	5.3	6.0	31.6
26	290	7.5	28.8	0.50	1.9	8.0	30.7
26	240	6.5	20.9	0.70	2.3	7.2	23.2
34	475	6.5	28.2	1.2	5.2	7.7	33.4
34	670	6.5	29.5	0.35	1.6	6.9	31.1
34	360	5.0	23.8	0.75	3.6	5.8	27.4
34	225	5.5	19.0	1.5	5.2	7.0	24.2
34	430	8.5	27.4	1.3	4.1	9.8	31.5
42	400	7.0	29.1	0.32	1.3	7.3	30.4
42	260	3.9	21.9	0.65	3.8	4.6	25.7
42	475	4.4	17.6	2.3	9.2	6.7	26.8
42	270	8.0	38.1	1.4	6.7	9.4	44.8
50	400	2.8	14.7	0.39	2.1	3.2	16.8
50	390	4.8	21.8	3.2	14.5	8.0	36.3
50	385	4.5	18.7	2.5	10.4	7.0	29.1
50	340	4.2	16.8	2.5	10.0	6.7	26.8
50	340	4.2	18.2	2.3	10.0	6.5	28.2
58	295	2.8	11.7	4.5	18.7	7.3	30.4
58	250	5.5	21.1	2.1	8.1	7.6	29.2
58	400	4.4	22.0	3.9	19.5	8.3	41.5
58	230	2.7	15.0	1.5	8.3	4.2	23.3
58	230	3.9	15.6	4.0	16.0	7.9	31.6

* Only a small fraction of this iodine is protein-bound.
† Refers to I^{127} as calculated from the radioactivity recovered in the gland.

after the first injection, escape occurred in all rats; their thyroids contained 2.3–3.2 gamma of *new* organically bound iodine.

DISCUSSION

The inhibitory action of excess iodide on organic-iodine synthesis in the thyroid gland has been confirmed here but shown to be temporary in nature. The maximum duration of this effect was 50 hours. Escape from inhibition was detected as early as 26 hours, even though plasma total iodine was maintained at levels at which inhibition was initially established. Since it is probably the inorganic iodine within the gland that is the immediate agent responsible for the inhibitory

effect,[6] it became pertinent to determine whether escape from inhibition after 26 hours occurred because the gland lost its capacity to hold high concentrations of inorganic iodide. Tables 3 and 4 show, however, that when escape occurred, the concentrations of inorganic iodide in the gland were as high as those which, in the earlier intervals, caused inhibition.

TABLE 5. REACCUMULATION OF ORGANIC IODIDE IN THYROIDS OF RATS TREATED WITH EXCESS IODIDE

(All rats were fed for the first 19 days a diet containing 0.2 per cent propylthiouracil. At the end of this period the first three rats were sacrificed. The remainder were fed a normal diet for nine or 15 days. Half of the animals of each group received by intraperitoneal injection 500 gamma of iodide as KI thrice daily.)

Time after propylthiouracil	I^{127} injected every 8 hours	Thyroid iodine			
		Inorganic	Organic		Total
			Nonthyroxine	Thyroxine	
days*	gamma	gamma	gamma	gamma	gamma
0	0		0.11†	0.22	0.33
0	0		0.14	0.16	0.30
0	0		0.11	0.27	0.38
9	0		0.81	0.17	0.98
9	0		0.63	0.22	0.85
9	0		2.2	0.54	2.7
9	500	5.4	4.8	0.71	10.9
9	500	7.2	1.8	0.40	9.4
9	500	3.0	4.0	1.0	8.0
15	0		2.2	0.45	2.7
15	0		4.3	0.83	5.1
15	0		2.6	0.70	3.3
15	500	4.0	3.8	0.75	8.6
15	500	5.0	3.6	0.45	9.1
15	500	1.2	4.6	1.1	6.9

* Normal rats of this group contained about 1.5 gamma of thyroxine and 4.2 gamma of nonthyroxine iodine.

† In the rats that received no iodide injections the inorganic iodine was not separated from the nonthyroxine fraction.

Although the problem of exchange has been discussed elsewhere (Chaikoff and Taurog, 1948), the fact that we are dealing here with higher concentrations of iodide leads us to reconsider it. It might be argued that prolonged exposure of the gland to high levels of inorganic iodide results in an exchange between inorganic I^{131} and organically bound I^{127}. If this were the case, the recovery of the I^{131} in the organic fraction (Tables 3 and 4) would not be indicative of renewed synthesis or escape. To test this possibility it was necessary to measure the accumulation of organic iodine in the thyroid gland without resorting to the use of I^{131}. The thyroids of rats were first depleted of their iodine stores by feeding 0.2 per cent propylthiouracil in the diet for 19 days. Thereafter, they were fed a normal diet and injected with excess iodide

[6] Cf. *in vitro* experiments by Morton *et al.* (1944).

(500 gamma every eight hours) for 15 days. As shown in Table 5, reaccumulation of organic I^{127} was not prevented by the excess iodide. In addition to confirming the temporary nature of the inhibitory action of excess iodide upon organic binding of iodine by the thyroid gland, these results lead us to conclude that exchange reactions could not account for the increase of organically bound $I^{1\,1}$ shown in Tables 3 and 4.

In view of the temporary nature of iodide inhibition in the normal rat thyroid, we must reopen the question as to whether a block in the formation of organic iodine is an adequate explanation for the characteristic action of excess iodine (Lugol's) in Graves' disease. An answer to this question must now await investigations in the human thyroid. The recent discovery by Stanley and Astwood (1948) that KSCN expels inorganic iodide from the thyroid offers, for the first time, a simple approach to this problem in man without surgical interference. In patients that have been injected with I^{131}, the inorganic iodide can be discharged from the thyroid gland by thiocyanate, and the organic I^{131} measured by external counting.

SUMMARY

Experiments designed to test the duration of inhibition, by excess iodide, on organic binding of iodine in the thyroid gland are described.

It is shown that the inhibitory action of excess iodide on the normal rat thyroid is temporary in nature. Inhibition lasted for about 26 hours. Despite the continued maintenance of a high level of plasma total iodine (100–200 gamma per cent), the formation of significant amounts of organic iodine was resumed after 26 hours.

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THE RELATIONSHIP OF THE MOUSE ADRENAL CORTEX TO THE PITUITARY

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The course of events in the mouse adrenal cortex after hypophysectomy has been dealt with (Jones, 1949; 1950). It seems that the zona glomerulosa is independent of the pituitary in so far as it may be considered a histological entity, and that both the zona fasciculata and the X zone degenerate after removal of the hypophysis. In this paper the results of the injections of different, albeit impure, fractions of the anterior pituitary into hypophysectomized mice are given.

The mice chosen are those whose adrenals possess an X zone and these fall into various categories. In the virgin female the X zone is a distinctive layer of cells surrounding the medulla and it persists for a varying length of time in adult life (Howard, 1927; Deanesly, 1928). In the male the X zone normally disappears at puberty. If, however, the immature mouse is castrated the X zone continues to develop and comes to occupy a large portion of the cortex as in the virgin female. The adult male mouse has no X zone, but postpuberal castration allows a new zone to arise around the medulla. This new cortical zone is very similar, histologically, to the X zone and it is termed the "secondary" X zone (Howard, 1939). These three categories of mice are considered, then, and the relationship of the various cortical zones to the pituitary is explored.

One injected substance is employed which is not of pituitary origin, namely, human chorionic gonadotrophin. This is used, firstly, because it has some properties in common with the luteinizing hormone of pituitary origin, and secondly, because it is a hormone of pregnancy. In the female mouse the X zone disappears during first pregnancy, and the factors effecting this are unknown. Although the hormone produced from the rodent placenta is not the same type as that from man (Astwood and Greep, 1938), nevertheless it was considered that the results from the use of human chorionic gonadotrophin might throw some light on the disappearance of the X zone in the primiparous female mouse.

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TABLE 1. TEST OF GONADOTROPIN PREPARATIONS USING HYPOPHYSECTOMIZED, IMMATURE MALE ANIMALS, FOUR IN EACH GROUP

	Body wt.	Testes	Seminal vesicles	Ventral prostate
(a) Mice				
Controls	14.3	79.6	6.1	1
Gonadotrophin I	12.8	100.6	24.1	4.3
(b) Rats				
Controls	43.2	140	9.5	7.4
Gonadotrophin I	38.2	391	18.7	35.9
Gonadotrophin II	44.9	368	17.0	25.7
Gonadotrophin III	43.5	225	11.2	24.1

MATERIAL AND METHODS

The general plan of the experiment was to take the various categories of mice which possess an X zone and after removal of the pituitary, to inject adrenocorticotrophin and gonadotrophins of several types, followed by examination of the adrenal glands. The spayed female was used in some cases in order to obviate possible hormonal mediation via the ovaries.

The mice, which were "Swiss" albino, were maintained on Purina Laboratory Chow or on a synthetic diet, with tap water *ad libitum*. Diet made no detectable difference to adrenal reactions. The advantage of the synthetic diet was that with its use the survival rate of animals after hypophysectomy is very good. The diet was made up from Shaw (1947) and a detailed analysis of its use is given in Shaw and Greep (1949) and see also Jones (1949).

The mice were killed with chloroform. The organs listed in Tables 1, 2 and 3 were dissected out and weighed on a Roller-Smith balance. The right adrenal and ovaries were fixed in Bouin's fluid, embedded in paraffin wax, cut at 7μ, and stained in Harris' hematoxylin and eosin or in Heidenhain's Azan. The left adrenal was fixed in 10 per cent neutral formalin and sectioned

TABLE 2. BODY AND ORGAN WEIGHTS OF VIRGIN FEMALE MICE IN THE DIFFERENT EXPERIMENTAL CATEGORIES

Experimental category	No. in group	Body wt.	Adrenals	Ovaries	Uterus	Thymus
1. Intact controls	20	19.7	6.8	5.6	44.1	60
2. Hyp. controls	20	18.7	3.0	2.5	8.2	54
3. Hyp. & Gon. I	7	19.2	—*	4.4	19.3	83
4. Spayed, hyp. & Gon. I	5	17.7	4.1	—	11.4	64
5. Spayed & Gon. I	5	22.6	8.3	—	12.9	71
6. Hyp. & Gen. II	5	18.2	—*	7.0	42.4	82
7. Spayed & Gon. IV	5	18.7	6.4	—	12.5	58
8. Spayed, hyp. & Gon. IV	5	17.2	3.5	—	11.9	74
9. Hyp. & Gon. IV	6	19.1	—*	4.4	17.5	72
10. Spayed, hyp., Gon. IV & estrogen	4	20.6	2.9	—	49.0	75
11. Spayed, hyp., Gon. IV & progesterone	2	18.7	3.7	—	31.7	33
12. Intact & ACTH	7	16.9	—*	3.8	28.7	20
13. Hyp. & ACTH	12	14.9	5.31	2.4	12.4	23

* Weights not taken

Abbrev.: Hyp. = hypophysectomized
Gon. = gonadotrophin preparation

at 15μ on the freezing microtome. These sections were treated routinely in the following manner: (1) Stained with Sudan IV; (2) stained with Sudan IV and counterstained with hematoxylin; (3) stained with Sudan black; (4) stained with Schiff's reagent; (5) mounted in glycerine and examined with the polarizing microscope and with the fluorescence microscrope; (6) extracted with acetone for $\frac{1}{2}$ hour at room temperature and examined as in (5), above; and (7) treated by the Schultz method for cholesterol.

The interpretation of the use of these histochemical methods in the mouse is discussed by Jones (1949; 1950). Suffice to say here that they are employed as indicative of acetone-soluble substances which seem to vary in expression with the activity of the adrenal cortex.

TABLE 3. THE ORGAN WEIGHTS OF POST-PUBERALLY CASTRATED MALE MICE, VARIOUSLY TREATED, COMPARED TO THOSE OF NORMAL INTACT MALES

	No.	Body wt.	Adrenals	Seminal vesicles	Thymus
Castrated	8	30.7	6.6	10.3	68
Castrated & hyp.	5	23.8	2.9	9.0	42
Castrated, hyp. & Gon. I or II	6	25.8	3.9	8.9	43
Intact normals	7	30.9	4.4	177.0	69

On the group with a secondary X zone in the adrenal, some further methods were employed. Reducing substances in the adrenal cortex were located by the method of Deane and Morse (1948). These reducing substances may well be mainly ascorbic acid. The method for the histochemical identification of alkaline phosphatase was that of Gomori (1939), using sodium β glycero-phosphate as a substrate. Values for "total" and "free" cholesterol in the adrenals were obtained by use of the modified Schoenheimer-Sperry method (Sperry, 1938).

The operation of hypophysectomy was performed by the parapharyngeal approach under "nembutal" anesthesia. The survival was good. The completeness of hypophysectomy was judged by the examination of serial sections of the appropriate area in several cases. In the remainder, the success of the operation was established by examination of the sella turcica area under the binocular microscope ($\times$15). Added confirmation was given, where relevant, by the organ weights and by the histological condition of the ovaries and adrenals. The term "hypophysectomy" is employed in this paper to mean that the anterior and intermediate lobes are removed (Newton and Richardson, 1940). Although the posterior lobe as such is removed, components above the diaphragma sella may substitute for it physiologically.

The preparations used in these experiments were as follows:

1) Gonadotrophin I. This was prepared from sheep pituitaries by Armour & Co., Lot FW-234. Besides gonadotrophin it contains, according to the makers: prolactin, 0.16 unit per mg.; thyrotrophin, 0.4 unit per mg.; growth, 0.2 Evans unit per mg. (epiphyseal disc method); adrenocorticotrophin, negligible; Posterior lobe, oxytocic (rooster), 0.2 unit per mg. and pressor (cat), 0.2 unit per mg.

The preparation was tested for its gonadotrophic properties by injection into hypophysectomized immature male rats and mice of 21–23 days of age

(see Table 1). The assumption is made that luteinizing hormone (LH; ICSH) stimulates the interstitial cells of the testis to produce androgens without causing sperm formation which is considered to be the function of the follicle stimulating hormone (FSH) (Greep, van Dyke and Chow, 1942). The ventral prostate in the rat is the most sensitive indicator of androgen activity, the seminal vesicles being less responsive; in the mouse they seem equally sensitive. From Table 1 it will be seen that the preparation is rich in LH but it is not pure in this respect. The increase in weight of the testes indicates that FSH is also present. The effect of this preparation on the hypophysectomized virgin female also indicated that some FSH was present, although LH activity was clearly dominant. The weight of the ovaries was nearly doubled as against the hypophysectomized controls, and the uterus weight was more than doubled (Table 2). Histologically the ovaries showed marked corpora lutea formation, whereas those of the hypophysectomized controls showed only small follicles.

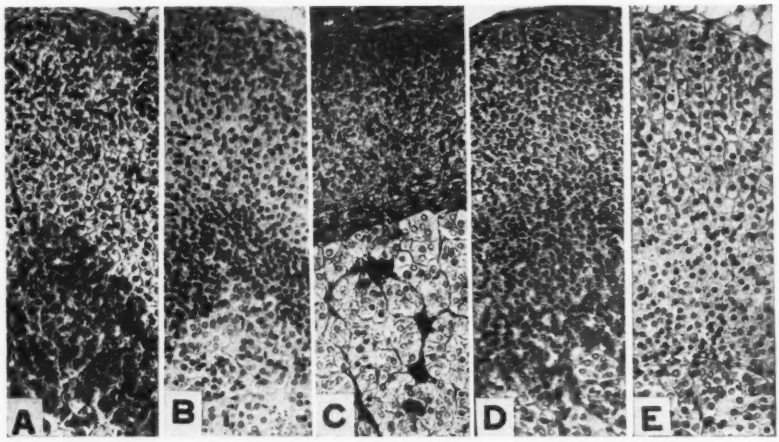

FIG. 1. Sections of adrenals of male mice. Bouin; Hemotoxylin and eosin except 1c which is stained with Heidenhains Azan.

(a) Normal immature male, 25 days old, showing the prominent X zone.
(b) Immature male, hypophysectomized for 5 days. The zona glomerulosa has a normal appearance, the zona fasciculata and the X zone are degenerating.
(c) Immature male, hypophysectomized for 5 days, injected with Gonadotrophin I for 4 days. The LH has caused the production of androgens from the testes which in turn have destroyed the X zone. A medullary connective tissue caspule has appeared.
(d) Castrated immature male, hypophysectomized for 5 days, and injected with Gonadotrophin I for 4 days. In this case no androgens are produced in the absence of the testes and the LH maintains the X zone in a condition approaching that of the normal.
(e) Young adult male. The androgens produced concomittant with puberty have destroyed the X zone, and a medullary connective tissue capsule is present. This effect was achieved in (c), above, in the hypophysectomized animal using exogenous LH.

The condition of the adrenals of the male mice used in the test of this

preparation is instructive. (Text-figure 1a-e) The removal of the pituitary causes the X zone and the zona fasciculata to degenerate, while the zona glomerulosa is little affected (1b). The X zone of the hypophysectomized male mice injected with gonadotrophin I has disappeared and a medullary connective tissue capsule formed (1c). This is due to the action of the androgens arising from the testes under the influence of LH. In the absence of the testes the gonadotrophin maintains the X zone of the hypophysectomized animal in a normal condition (1d).

2) Gonadotrophin II. This pituitary preparation is the "Synergist" of Schering Corporation. Its gonadotrophic properties were tested by use of hypophysectomized immature rats. The preparation contains both FSH and LH properties as evinced by the increase in testes weight and in the prostate as compared with the hypophysectomized controls (Table 1). When injected into hypophysectomized virgin females (Table 2), the ovaries increased in weight almost threefold, and the uterus more than fivefold as compared with the hypophysectomized controls. Histologically the ovaries of the injected animals showed marked follicular development with some corpora lutea. It would appear that Gonadotrophin I and II show both FSH and LH activities. It is probable that Gonadotrophin I is predominantly LH in nature and Gonadotrophin II predominantly FSH.

3) Gonadotrophin III. This pituitary preparation is that of Dr. W. H. McShan, Lot LH 15 HM3. Assayed, using immature hypophysectomized male rats (Table 1), both LH and FSH activities were shown. It would seem that the LH potency is greater than the FSH in so far as the prostate weight is increased nearly fourfold but the testes weight not twofold as compared with the hypophysectomized controls. Also, in comparison with Gonadotrophin II, the LH potency is much the same but the FSH activity of the McShan preparation is much less.

4) Gonadotrophin IV (HCG). This is a commercial preparation of human chorionic gonadotrophin ("Korotrin"). Injected into hypophysectomized virgin females the weight of the ovary and of the uterus were increased (Table 2). Histologically the ovaries showed interstitial cell and theca interna growth, with no corpora lutea. This and the relatively small increase in the weight of the unterus would indicate the predominantly "LH-like" nature of HCG, without a significant amount of "FSH-like" activity.

5) Adrenocorticotrophin (ACTH). Two lots of Armour & Co. ACTH were used, Lot 42B and Lot 37KE. Lot 42B assayed for ACTH activity at 16 $\pm$ per cent of Armour standard La-1-A (see Forsham et al., 1948). It contained impurities, mainly: posterior pituitary hormone, oxytocic, 0.0036 unit per mg. and pressor, 0.0045 unit per mg.; prolactin, 1 unit per mg.; gonadotrophins, 1.5 Collip units per mg; growth, a negligible amount. Lot 37KE had 41.5 $\pm$ 12 per cent of the Armour standard activity. The chief impurities consisted of oxytocic, 0.0025 unit per mg., pressor, 0.005 unit per mg.; prolactin, 0.5 unit per mg.; gonadotrophins, 2.0 Collip units per mg.; growth, a negligible amount.

Both preparations were potent ACTH preparations in the mouse. Judged by their effect on the hypophysectomized virgin female the gonadotrophin content is nil, the ovaries showed no stimulation histologically or by weight, and the uterine weight was well within the range of that of the hypophysectomized female (Table 2). In this paper animals treated with the two dif-

ferent preparations are not differentiated as it is concerned only with qualitative histological differences.

The estrogen used was α estradiol benzoate (Ben Ovocylin, Ciba), and the progesterone, Lutocylin (Ciba).

Details of Treatment

I. The X zone in the virgin female.

The different experimental categories are shown in Table 2. The mice were virgin females, aged 40–45 days, at the commencement of the experiment and were killed 12–14 days later. In all the hypophysectomized animals injections were started 24 hours after operation and killed 24 hours after the last injection. One mg. of Gonadotrophin I and of Gonadotrophin II was injected per day in each case. Injections were given subcutaneously, once per day for 12 days. Gonadotrophin III was not used with this group. Gonadotrophin IV was given in daily intraperitoneal injections of 11 I. U. for 10 days. In Category 10, Table 2, in addition to the Gonadotrophin, 1 R. U. of estrogen was given, subcutaneously for the same length of time. In Category 11, Table 2, 1.5 mg. of progesterone per day were given under the same conditions. Adrenocorticotrophin was given in the dose of 1 mg. per day, divided into three intraperitoneal injections, for 10–12 days.

II. The X zone in the prepuberally castrated male mouse.

A group of 7 males were castrated at 21–23 days of age. Twelve to 14 days later, during which time the X zone has continued to develop, they were hypophysectomized. Twenty-four hours after operation injections of Gonadotrophin I were started, continued for 7 days, and the animals killed 24 hours after the last injection. The castrated controls, and the untreated hypophysectomized castrates have been described in detail by Jones (1949) to which reference should be made.

III. The secondary X zone of the postpuberally castrated male mouse.

The different experimental categories are shown in Table 3. Adult male mice, taken from 5 litters born within a day of each other were castrated at the age of 2½ months. The experiment was commenced 2 months later when the secondary X zone was well developed. Three hypophysectomized animals were injected with 1 mg. of Gonadotrophin I per day, subcutaneously for 16 days. Injections were started 24 hours after operation, and the animals killed 24 hours after the last injection. Under the same conditions 1.5 mg per day of Gonadotrophin III were injected into 3 hypophysectomized animals. At these dosage levels the LH activity of the two preparations was estimated to be about the same (see Table 1). The organ weights of the hypophysectomized animals injected with these two preparations are shown together in Table 3 as there was no obvious difference between the two sets.

RESULTS

Intact controls. The adrenal of the Swiss albino virgin female mouse, aged between 40 and 60 days, consists of an outer connective tissue capsule, zona glomerulosa, outer and inner zona fasciculata, X zone and the medulla (fig. 1). The zona fasciculata abuts directly

onto the X zone without the intervention of a zona reticularis. There is no medullary connective capsule. The X zone is an obvious layer of cells when seen in sections stained in hematoxylin and eosin preparations after Bouin's fluid. The cytoplasm is very eosinophil, the nuclei large, round and basophilic. At these ages the X zone is well developed and shows none of the spontaneous "fatty" degeneration which occurs later in life (Howard, 1927). In the sections prepared after the histochemical methods, the zona glomerulosa and zona fasciculata are sudanophilic (fig. 6), Schiff positive (fig. 10), give the Shultz cholesterol reaction, have a stable greenish-blue fluorescence in ultraviolet light, and contain birefringent particles (fig. 14). The cytoplasmic droplets produce these reactions except perhaps in the case of the birefringent particles (Yoffey and Baxter, 1947), although other observations (Deane and Jones, unpublished) would indicate that the birefringence originates in these droplets for the most part. Positive reactivity is removed by acetone extraction. The overall picture shows that the zona glomerulosa in the Swiss albino is less reactive than the zona fasciculata, that is, the cytoplasmic droplets are less numerous in the zona glomerulosa. The X zone does not contain substances which react with the Sudans, or the Schiff reagent or give a positive reaction with the Schultz test. No birefringent particles are formed in the zone with or without formalin fixation. The fluorescence is a nonspecific bluish-black common to many tissues of the body. The medulla, which does not concern this paper, is not reactive to any of these tests.

The hypophysectomized virgin female. After removal of the pituitary, both the zona fasciculata and the X zone degenerate (Jones, 1949; 1950). Twelve to 14 days after operation the nuclei of the zona fasciculata have become pyknotic and the cell cytoplasm has shrunken (fig. 2). The cytoplasmic droplets have coarsened and coalesce in parts

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FIG. 1. Normal virgin female mouse, age 55 days. The juxtamedullary X zone stands out as a wide deeply stained layer, contrasting with the rest of the cortex.

FIG. 2. Virgin, hypophysectomized at 40 days of age. Length of time hypophysectomized, 14 days. The zona glomerulosa is not markedly different from that of the control. The degeneration of the zona fasciculata is well advanced. Areas of degeneration are particularly apparent in the region of the inner zona fasciculata and the outer part of the X zone. The X zone has collapsed and is represented by a layer of pynotic nuclei lying against the medulla.

FIG. 3. Virgin, spayed at 43 days, hypophysectomized at 45 days. Length of time hypophysectomized, 14 days. Injections of Gonadotrophin I for 12 days. The X zone is maintained with the nuclei normal in appearance and the cell cytoplasm staining with eosin. The zona glomerulosa is normal in appearance. The zona fasciculata is degenerating and narrow.

FIG. 4. Virgin, hypophysectomized for 14 days. Injection of adrenocorticotrophin for 12 days. The zona fasciculata is wede and well maintained. The X zone is collapsed, its nuclei pyknotic, the cell cytoplasm shrunken and non-eosinophitic. The zona glomerulosa has a normal appearance.

FIG. 5. Virgin, hypophysectomized for 12 days. Human chorionic gonadotrophin for 10 days. The X zone has disappeared and a medullary connective tissue capsule is present. There is a tendency towards hyperemia.

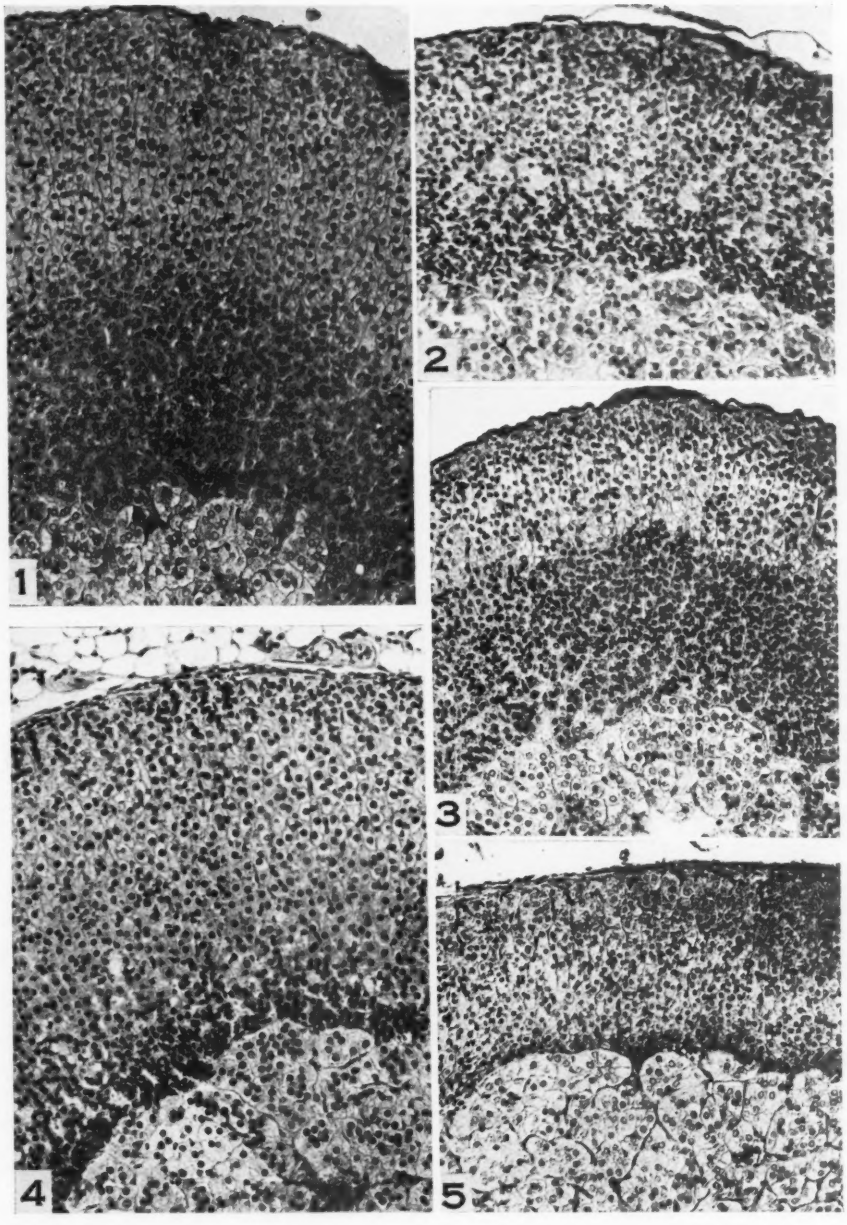

PLATE I. Sections of mouse adrenals. Harris' haemotoxylin and eosin. ×180.

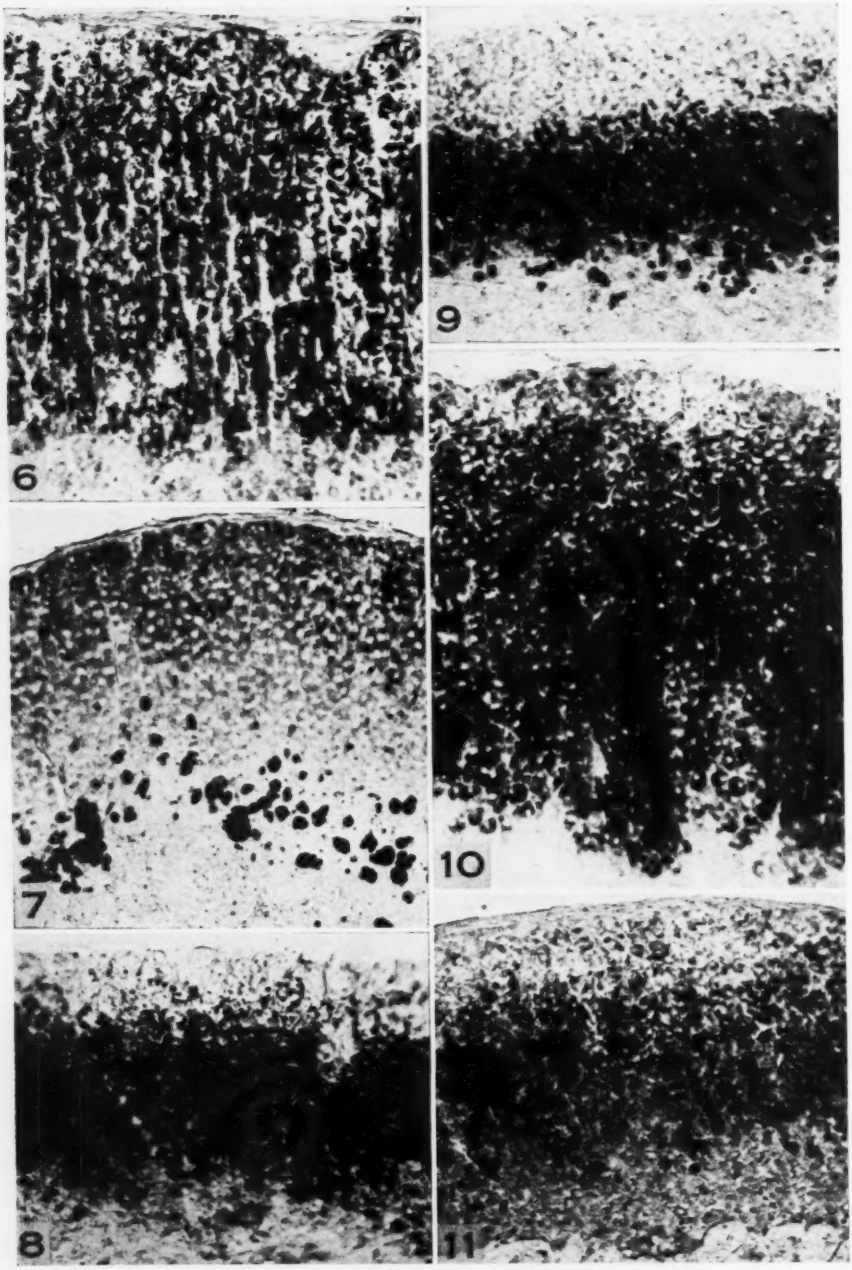

PLATE II. Sections of the adrenals of virgin female mice cut on freezing microtome at 15 μ. Figures 6–9 stained with Sudan IV. Figures 10 and 11 with the Schiff reagent. ×180

especially toward the inner region of the zona fasciculata where actual lobules of sudanophilic material appear. The staining with Sudan is more intense (fig. 7), and the Schiff reagent (fig. 11) gives a deeper color when compared to that in the normal animal. The greenish-blue fluorescence has vanished and only the nonspecific blue-black remains. The birefringent particles have become larger, and there is a tendency to clump together (fig. 15). The X zone has lessened in area, the nuclei have become pyknotic, the cell cytoplasm considerably reduced and the zone is no longer eosinophilic (fig. 2). The X zone is still not reactive to the histochemical tests, except that sudanophilic degenerative areas may have appeared, especially in the outer part (fig. 7). The changes in the adrenal are no different when the animal is spayed 48 hours before removal of the pituitary. The zona glomerulosa, on the other hand, does not degenerate after hypophysectomy and, in the histological preparations, differs little in appearance from that of the normal. The zone, however, by 14 days after operation, shows little to no sudanophilic, Schiff positive, and Schultz positive material; birefringent particles are rare and it shows only the blue-black fluorescence.

The hypophysectomized virgin given injections of Gonadotrophin I. After injections of Gonadotrophin I for 12 days, the adrenal presents a different appearance. In histological preparations both the zona glomerulosa and the zona fasciculata are much the same as in the untreated hypophysectomized animal (fig. 3). The X zone does not show the typical degeneration, but the cells are well maintained, the nuclei round, the cytoplasm plentiful and eosinophilic. There are no degenerative areas to be found therein. The zone itself is wide

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FIG. 6. Normal, aged 60 days. The sudanophilia of the zona glomerulosa and the zona fasciculata is apparent, and is confined to the cytoplasmic droplets. Only a portion of the X zone is visible, at the bottom of the photograph. It is not sudanophilic. The medulla is not shown.

FIG. 7. Virgin, hypophysectomized for 14 days. Adrenocorticotrophin for 12 days. The sudanophilia of the zona glomerulosa and the outer region of the zona fasciculata may be seen and this lessons toward the inner zona fasciculata. Lobules of degeneration can be seen in the inner region of the zona fasciculata and in the X zone.

FIG. 8. Virgin female hypophysectomized for 14 days. Much of the sudanophilia has disappeared from the zona glomerulosa. The zona fasciculata is densely stained, and the droplets have coarsened and coalesced. A few sudanophilic lobules can be seen in the X zone at the bottom of the photograph.

FIG. 9. Virgin hypophysectomized for 14 days. Gonadotrophin I for 12 days. The absence of sudanophilic material from the zona glomerulosa is particularly marked. The zona fasciculata presents the typical degenerated appearance after hypophysectomy. The X zone in these injected animals is well maintained. This is not apparent in the illustration as the zone is not sudanophilic.

FIG. 10. Normal virgin. The zona fasciculata has reacted strongly with the Schiff reagent, the zona glomerulosa less so. The X zone does not react, a small portion is to be seen at the bottom of the photograph.

FIG. 11. Virgin, hypophysectomized for 14 days. The zona glomerulosa reacts weakly. The degenerating zona fasciculata shows varying strnegth of reaction. The degenerating X zone is now reactive.

and approaches that of the normal (cf. fig. 3). The pictures after Sudan (fig. 9), Schiff (fig. 12), the fluorescence and birefringence (fig. 16) are similar to that of the untreated hypophysectomized animal. The maintained X zone is not reactive to the histochemical tests used, following the zone of the normal adrenal in this respect.

The spayed, hypophysectomized female injected with Gonadotrophin I. Spayed, hypophysectomized females injected with this preparation possessed a well-maintained X zone, approaching that of the normal in histological appearance. The undegenerated zona glomerulosa, the regressing zona fasciculata and the X zone of normal appearance can be clearly seen in the illustration (fig. 3). There is no significant difference between the hypophysectomized animal injected with Gonadotrophin I and the spayed with similar treatment. This seems to indicate that the action of the Gonadotrophin is a direct one on the X zone, without ovarian mediation. Illustrations in figures 9, 12 and 16 serve to represent both categories.

The spayed female (not hypophysectomized) injected with Gonadotrophin I. No histological or histochemical difference could be detected between the adrenals of these animals and those of the normal mice. There is an indication in the treated animals that the X zone is of larger area. This is an impression and the numbers are too small for statistical confirmation.

The hypophysectomized female injected with Gonodotrophin II. After this treatment, the zona glomerulosa and the zona fasiculata were similar to those of the untreated hypophysectomized controls. On the other hand, the degeneration of the X zone had been fairly

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FIG. 12. Virgin, hypophysectomized for 14 days, Gonadotrophin I for 12 days. Little reaction in the zona glomerulosa, moderate in the zona fasciculata, with some degenerative areas giving a strong reaction. The X zone, which is well maintained in these animals, does not react, but the faint cytoplasmic wash brings it out in the photograph. The medulla lies to the bottom of the photograph.

FIG. 13. Virgin, hypophysectomized for 14 days. Adrenocorticotrophin for 12 days. The zona glomerulosa and the outer region of the zona fasciculata react strongly, the inner zona fasciculata less so. The narrow collapsed X zone is non-reactive.

FIG. 14. Normal virgin. A mixture of fine and coarse birefringent particles are scattered in the zona glomerulosa and the zona fasciculata, although particles are less prevalent in the zona glomerulosa. The X zone does not show as it does not contain birefringent material.

FIG. 15. Virgin, hypophysectomized for 14 days. The outer connective tissue capsule shows in part due to its intrinsic birefringence as do some of the radial connective tissue fibers of the cortex. The zona glomerulosa has few birefringent particles in it. The zona fasciculata possesses mainly coarse clumps of birefringent material.

FIG. 16. Virgin, hypophysectomized for 14 days. Gonadotrophin I for 12 days. The picture is similar to 15 above, indicating that the gonadotrophin had no effect on the zona glomerulosa and the zona fasciculata. The non-degenerated X zone does not show as it contains no birefringent particles.

FIG. 17. Virgin, hypophysectomized for 14 days, adrenocorticotrophin for 12 days. A mixture of fine and coarse birefringent particles are present in the zona glomerulosa and in the outer zona fasciculata. The inner zona fasciculata contains few such particles. The degenerative lobules in the inner part of the zona fasciculata and the X zone show a faint fine birefringence.

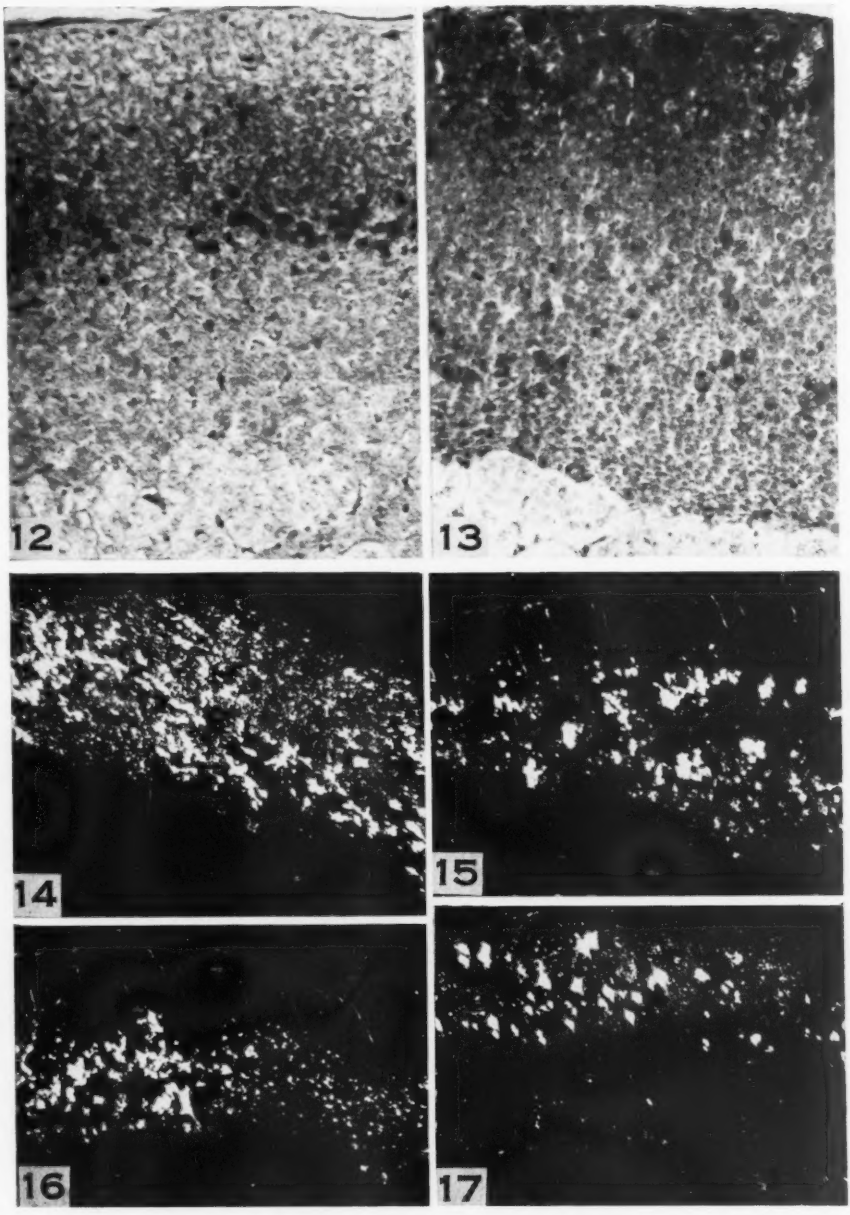

PLATE III. Sections of adrenal of virgin female mice, cut on the freezing microtome at 15 μ. Figures 12 and 13 after the Schiff reagent. ×180. Figures 14–17 mounted plain in glycerine, and viewed with the polarizing microscope. ×120.

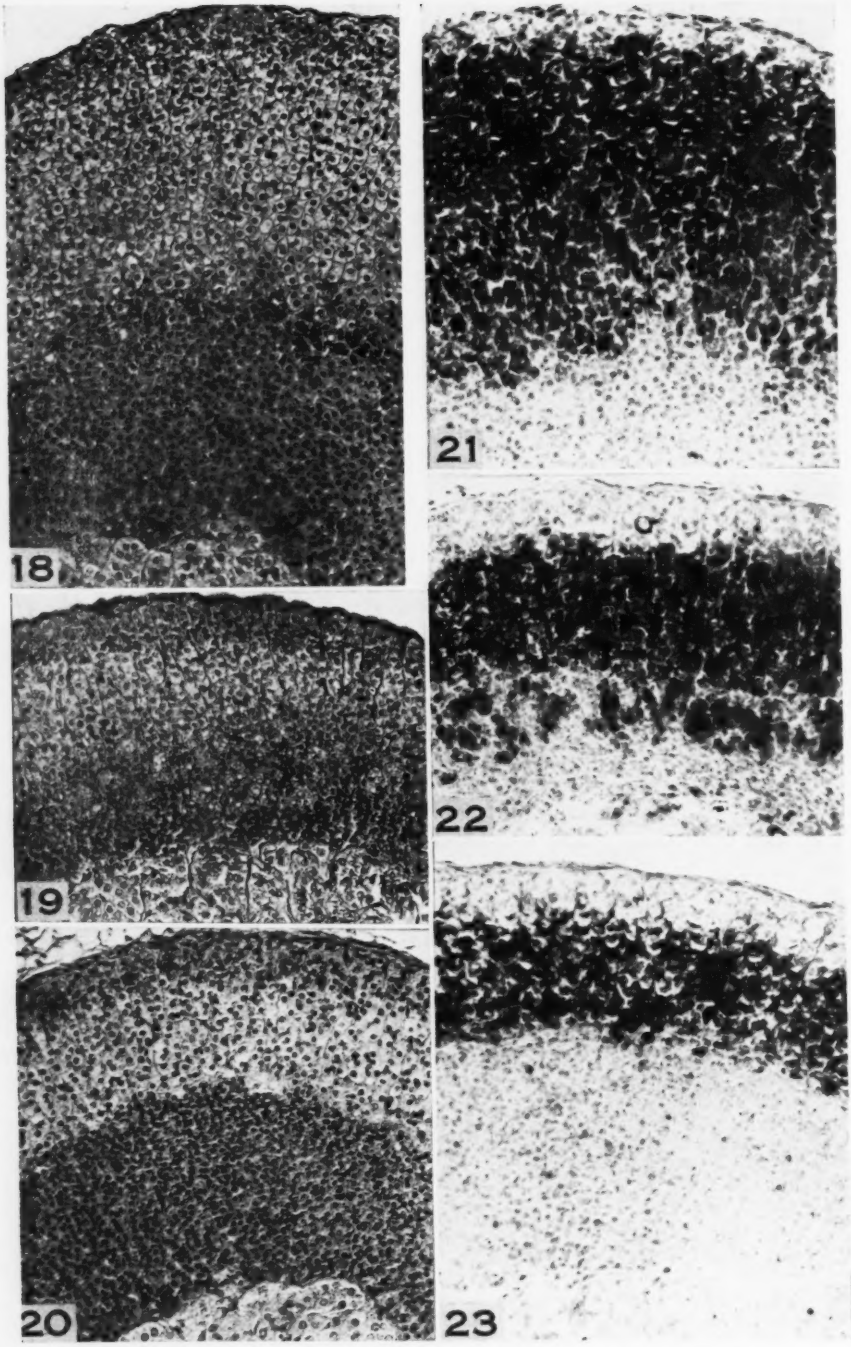

PLATE IV. Adrenals of postpuberally castrated male mice. Figures 18–20 hemotoxylin and eosin. Figures 21–23, frozen sections of 15 μ, stained with Sudan IV.

well prevented. The maintenance, however, was not so complete as in those animals injected with Gonadotrophin I. For the most part those cells nearest the medulla were normal, while those more peripherally placed showed signs of degeneration.

The intact female injected with adrenocroticotrophin (ACTH). There is an overall increase in the size of the adrenal cortex which is apparent by inspection, although the adrenals were not weighed. The zona glomerulosa and the zona fasciculata become filled with fine sudanophilic droplets which are Schiff and Schultz positive. The fluorescence is a greenish-blue, spreading over the two zones. The birefringent particles are fine and scattered plentifully in the same region. The increase in size of the cortex is due to the contributions of the zona glomerulosa and the zona fasciculata. The X zone is, in general, narrower than that of the controls. The cells of this zone are in nowise degenerate however. It seems likely that the X zone becomes more compact after ACTH injections, due to the mechanical pressure exerted by the enlargement of the rest of the cortex.

The hypophysectomized female injected with ACTH. In the adrenal of the hypophysectomized female injected with *ACTH* the zona fasciculata is maintained so that it has all the histological characteristics of that of the normal animal. The zona glomerulosa has not altered its appearance in histological preparations (fig. 4). The histochemical preparations are of interest. Sudanophilic material is maintained in the zona glomerulosa (fig. 7), and this is both Schiff (fig. 13) and Schultz positive. The zone gives a greenish-blue fluorescence and numerous fine birefringent particles are to be found in it (fig. 17). The reactions are strongly positive, more so than in the untreated normal animal but similar to those of the normal treated with ACTH. The

FIG. 18. Untreated castrate. The zona glomerulosa and zona fasciculata are typical. There is no zona reticularis. The secondary X zone which has appeared consequent on castration is wide and deeply staining. A small portion of the medulla is to be seen at the bottom of the photograph.

FIG. 19. Castrate, hypophysectomized for 21 days. The cells of the zona glomerulosa are packed together but have not degenerated. The zona fasciculata is degenerating with vacuolated regions at its inner border and the outer part of the secondary X zone. The secondary X zone has collapsed. the nuclei are pycnotic and there is little cell cytoplasm. The medulla may be seen at the bottom of the photograph.

FIG. 20. Castrate, hypophysectomized for 21 days, Gonadotrophin I for 19 days. The zona glomerulosa is normal, the zona fasciculata is narrow and degenerating. The secondary X zone is wide and well maintained.

FIG. 21. Castrate. The zona glomerulosa is not as strongly sudanophilic as the zona fasciculata. The secondary X zone is not sudanophilic.

FIG. 22. Castrate, hypophysectomized for 21 days. Sudanophilic material has completely disappeared from the zona glomerulosa. The narrow zona fasciculata is densely stained. Lobules of degeneration can be seen in the inner zona fasciculata and in the X zone.

FIG. 23. Castrate, hypophysectomized for 21 days, Gonadotrophin III for 19 days. The zona glomerulosa and zona fasciculata are much the same as in figure 22, above, the hypophysectomized castrate. The zona fasciculata has a narrower and neater appearance perhaps due to the mechanical pressure of the X zone. This latter zone is wide and well maintained.

zona fasciculata reacts similarly to the histochemical tests as that of the normal. The sudanophilic droplets are generally fine, however. In some cases the sudanophilia disappears from the inner region of the zona fasciculata and this condition is illustrated in figure 8. The course of the degeneration of the X zone after hypophysectomy is unaltered by ACTH injections. The lobules of intensely staining sudanophilic material seen in figure 7 arise from the degenerating X zone and, also from cells of the inner part of the zona fasciculata. The degenerating X zone in the ACTH injected animals is more compact than that of the untreated hypophysectomized animal. This may be due to the mechanical pressure of the maintained zona fasciculata.

The spayed female (not hypophysectomized) injected with Gonadotrophin IV (HCG). Gonadotrophin IV (human chorionic gonadotrophin) injected into the spayed female had no detectable effect on the adrenal. Human pregnancy urine fraction or HCG, when injected into the intact virgin mouse, will cause the X zone to degenerate (Takewaki, 1936; Waring, 1942).

The spayed, hypophysectomized female injected with HCG. The adrenals of the mice in this category appeared no different from those of untreated hypophysectomized controls. It would seem that there is no direct action of this gonadotrophin on the adrenal cortex.

The hypophysectomized female injected with HCG. In the adrenals of the mice in this group, the X zone had disappeared and a medullary connective tissue capsule formed[2] (fig. 5). In the other two zones, the zona glomerulosa and the zona fasciculata, no difference from the course of events after hypophysectomy of the untreated was observed. This indicates that the action of this Gonadotrophin is one on the ovaries without the influence of the animals' own pituitary.

The spayed, hypophysectomized female injected with HCG and with estrogen. The X zone followed the same pattern of degeneration as in the untreated hypophysectomized female, apparently uninfluenced by the preparations injected. Estrogen alone in the same dosage was without effect. The rest of the adrenal was similar to that of the untreated hypophysectomized controls. It would seem that the substance produced from the ovary by HCG which causes X zone destruction is not an estrogen or the synergestic action of the two compounds. The dosage of 1 RU per day may be inadequate. On the other hand, it is doubtful if even large doses of estrogen would cause destruction of the X zone when given over only a short period of, say, 10 days (literature in Waring, 1942; Leathern and Silverman, 1945; Parkes, 1945). Longer periods of treatment would not be comparable

[2] It may be noted that a primary X zone and a medullary connective tissue capsule do not exist in one and the same adrenal. Normally, in the male, as the X zone disappears with puberty, a medullary connective tissue capsule forms; and, *mutatis mutandis*, in the adrenal of the primiparous female. A medullary connective tissue capsule, however, coexists with the secondary X zone.

to the normal rapid degeneration of the X zone, usually by the 12th day of first pregnancy.

The spayed, hypophysectomized female injected with HCG and progesterone. In these animals the X zone has not disappeared. Progesterone itself in the dosage used does not cause X zone degeneration and does not, even in larger doses, reproduce the effect of pregnancy on the X zone (Martin, 1930, Tolenaar, 1939; Howard and Gengradom, 1940; Waring, 1942; McPhail and Read, 1942; Clausen, 1944). It seems that there is no synergism between HCG and progesterone. Hence, the substance evoked by HCG from the ovaries of the intact or hypophysectomized virgin female is probably neither estrogen or progesterone.

The prepuberally castrated male mouse, normal and hypophysectomized. Castration of the immature male mouse prevents the degeneration of the X zone as the mouse reaches adult age; moreover, the X zone grows in size until it comes to occupy a large percentage of the cortex, as in the virgin female. Such castrates, hypophysectomized at 36 days of age when the X zone has become prominent, show degeneration of the zona fasciculata and X zone by 9 days after operation. The zona glomerulosa presents the same appearance as that of the virgin female described above. This aspect of the prepuberally castrated male mouse has been described and figured in Jones (1949) to which reference should be made. The progress of events after hypophysectomy are no different from those described for the virgin female in this paper with the proviso that the changes are not so far advanced, since the castrates were killed 9 days after operation as against 14 for the virgins.

The hypophysectomized prepuberally castrated male mouse, injected with Gonadotrophin I. This category of mouse injected with the preparation for 7 days shows a maintenance of the X zone, with degeneration of the zona fasciculata and the histochemical changes in the zona glomerulosa in much the same way as in the normal female. No separate illustrations are given, therefore, as they resemble closely figures 3, 9, 12, and 16.

The postpuberally castrated male mouse. Castration of the adult male mouse results in an enlargement of the gland so that 2 months after castration of $2\frac{1}{2}$-month old males, i.e., at $4\frac{1}{2}$ months of age, the pair of adrenals is 51.1 per cent heavier on the average than those of coeval intact males (Table 3). Histological preparations (fig. 18) reveal that an additional layer of cells have appeared surrounding the medulla, and these cells are very similar to those of the X zone, and is termed the "secondary X zone" (Howard, 1939). The adrenal of the normal adult male mouse shows little zona reticularis or none at all (Jones, 1948). It is probable, although not proven, that the new adrenal zone, which arises as a result of castration, is formed by prolifer-

ation of the cells of the inner region of the zona fasciculata where it abuts onto the medullary connective tissue capslule.

The zona fasciculata is strongly sudanophilic, the zona glomerulosa rather less so (fig. 21). The sections prepared after the Schiff reagent, the Schultz method, and for birefringeence and fluorescence are very similar to those of the female (figs. 10 and 14), and they are not, therefore, separately figured. The secondary X zone is not sudanophilic, not Schiff positive, nor Schultz positive. It contains no birefringent particles and gives a nonspecific blue-black fluorescence. The adrenal of the postpuberally castrated male mouse some time after castration is, therefore, very similar to that of the young adult virgin female. In the former, however, a medullary connective tissue capsule is present coexisting with the secondary X zone (see footnote 2).

The postpuberally castrated, hypophysectomized male mouse. Hypophysectomy of the castrated male with a well-developed secondary X zone results, by 18 days after operation, in adrenal weight loss of 56 per cent (Table 3). Histological preparations show that the zona glomerulosa is well maintained and that there is considerable degeneration of the zona fasciculata and secondary X zone (fig. 19). After the Sudan stains, the reactions noted as occurring after hypophysectomy in the virgin female, are to be seen (fig. 22), and the pattern after the Schiff reagent, the Schultz method, and with the fluorescence and polarizing microscopes is very similar.

The postpuberally castrated, hypophysectomized mouse injected with Gonadotrophin I or with II. The zona glomerulosa and the zona fasciculata are similar to those zones in the untreated hypophysectomized animal (figs. 20 and 23). The X zone, in contrast to that of the untreated, consists of cells normal in appearance, with round nuclei and eosinophilic cytoplasm (figs. 20 and 23). It is similar in appearance and gives the same reactions as that of the castrate with intact pituitary. This differential maintenance of adrenal zones is reflected in adrenal weights (Table 3). The hypophysectomized animals injected with gonadotrophin have adrenals which are 24.2 per cent heavier than the untreated hypophysectomized animals.

There are some additional points of interest in connection with the postpuberally castrated group of mice. Comparison of the seminal vesicle and ventral prostate weights of the untreated castrates with the normal (Table 3) shows that the former have regressed considerably. There is no evidence from this, therefore, that the adrenal of the castrate animal (and, by inclusion, the secondary X zone) produces a steroid with androgen-like properties. Further, the hypophysectomized animal with the X zone supported by gonadotrophin injections shows no seminal vesicle or prostate stimulation. Another line of evidence comes from the use of the histochemical method of demonstrating alkaline phosphatase. The adrenals of the castrated

male (and of the hypophysectomized and gonadotrophin injected animals) do not show any alkaline phosphatase. The normal male adrenal does so, and the alkaline phosphatase reaction can be brought back to the cortex of the castrate by injections of androgens (Elftmann, 1947). From this it appears that the absence of alkaline phosphatase from the adrenal cortex of the castrated male mouse would indicate that there is no androgen formation.

The estimation of cholesterol (Table 4) shows that in mg per cent, the adrenals of the normal animal and the castrate have about the

TABLE 4. THE MEAN CHOLESTEROL CONTENT OF THE ADRENALS OF NORMAL ADULT AND COEVAL POSTPUBERALLY CASTRATED MALE MICE, CASTRATED FOR TWO MONTHS

	No.	Adrenal wt. mean of pairs	Cholesterol content		
			Total absolute	Total	Free
			mg.	mg. %	mg. %
Normal	5	3.7	.054	1.39	.69
Castrated	5	6.9	.095	1.37	.86

same amount of total and free. In absolute quantities the adrenal of the castrate has about twice as much total cholesterol content as the normal. Since the histochemical preparations indicate that the secondary X zone does not contain cholesterol, it seems likely that, although the permanent cortex of the castrate may have enlarged also, there is in addition a higher cholesterol content in the zona glomerulosa and zona fasciculata as compared to those of the normal.

The one histochemical property which the secondary X zone has in common with the rest of the cortex, is the presence of "ascorbic acid" in the cells. The untreated castrate adrenal, prepared after the method of Deane and Morse (1948), shows fine black granules in the cells of all three zones. The adrenal of the hypophysectomized castrate shows the fine granules in the zona glomerulosa and occasional coarse clumps in the degenerating zona fasciculata and X zone. The adrenal of the hypophysectomized castrate injected with gonadotrophin shows fine black granules in the cells of the zona glomerulosa and the X zone; the zona fasciculata has scattered coarse black clumps. The interpretation of this finding is not clear. It does bring out the point that the gonadotrophin seems to maintain the X zone in a normal condition. The reducing substances revealed may be ascorbic acid, but the connection of this substance with adrenal cortical physiology is conjectural (Long, 1946).

DISCUSSION

The traditional zoning of the mammalian adrenal cortex on histological grounds may well be paralleled by divisions of physiological

function. In the mouse at least, it is clear that three zones, the zona glomerulosa, the zona fasciculata and the X zone have different relationships with the pituitary gland. The zona glomerulosa does not degenerate after removal of the pituitary. Indeed, it continues to divide so that in the long-term hypophysectomized animal the zone is quite wide (Jones, 1950). Although this property is independent of the hypophysis, nevertheless, the zone is not completely free of pituitary control. In the ACTH injected, hypophysectomized animal, acetone-soluble material (which is sudanophilic, Schiff and Schultz positive, possesses a specific fluorescence and is birefringent) is plentifully scattered in the zona glomerulosa. In the untreated animal this material disappears from the zone. It is apparent from the discussion of the tests used (Dempsey, 1948; Jones, 1949, 1950; Yoffey and Baxter, 1949) that they cannot be taken as rigorously specific for one class of compound. They reveal substances, nevertheless, related to the hormonal activity of the gland. It would follow, therefore, that adrenocorticotrophin plays a role, as yet undetermined, in the regulation of the zona glomerulosa.

While the complete dependence of the zona fasciculata on ACTH requires no comment, the X zone adds yet another variant to the pituitary-adrenal relationship. It is apparent that the factor trophic to the latter zone is not ACTH but a gonadotrophin. The gonadotrophic preparations used were not pure so that it is impossible to say whether luteinizing hormone (LH), or follicle stimulating hormone (FSH), or a synergistic reaction between the two, is responsible for the effect on the X zone. It would seem that the balance of evidence is in favor of considering the X zone-trophic factor as LH. Certainly the degree of maintenance of the X zone was directly related to the LH potency of the preparation employed. Further, Greep and Jones (1950) showed that rat pituitaries, LH in nature, maintained the X zone when injected into immature hypophysectomized female mice while rat pituitaries, FSH in type did not.

If the X zone maintaining factor is LH then it seems that in the male the androgens produced by the gonadotrophin from the interstitial cells act directly on the zone (Jones, 1949). Their destructive action over-rides the maintaining effect of the LH. If, however, the animal is castrated before puberty then the pituitary LH continues to act on the X zone, without the intervention of androgens, causing its growth. In the virgin female the increase in size of the X zone up to puberty and its persistence beyond is not surprising, if the zone is dependent on LH. The reason for its gradual degeneration later in adult life is not clear. It is possible that there is a lessening of LH secretion with age, although a sufficient level is maintained for the continuance of the estrous cycle. It is doubtful if androgens of ovarian origin are responsible because the course of degeneration is unaltered by ovariectomy (Howard, 1927; Deanesly, 1928). The possibility

exists that the adrenal steroid hormones may have a very slight androgenic effect sufficient to cause gradual degeneration of the zone.

The mechanism of the rapid degeneration of the X zone during first pregnancy is not known. Efforts to attribute this to estrogen or to progesterone or to their concerted action have failed (Howard and Grengradom, 1940; Waring, 1942). Furthermore, the X zone of the pseudopregnant animal—achieved by sterile mating (Takewaki, 1939) or by luteotrophin (Jones, unpublished)—does not degenerate. Nor, apparently, does the formation of deciduoma affect the zone (Takewaki). Removal of the embryos however, at the relevant time prevents or arrests the X zone degeneration (Takewaki, 1936). The experimental use of HCG in this respect may not be relevant to the mouse as the rodent placenta does not produce this type of gonadotrophin (Astwood and Greep, 1938). The difference in action between HCG, which has some luteinizing hormone properties, and LH, is marked. The former produces a substance from the mouse ovary, without pituitary mediation, which acts directly on the X zone to cause it to degenerate. This substance is probably neither estrogen or progesterone. Takewaki (1935) suggested that a third ovarian hormone accounts for this and Starkey and Schmidt (1938) that an ovarian androgen is responsible. In the consideration of the degeneration of the zone during first pregnancy some confusion has arisen because it is generally believed that implantation is *not* necessary for this to occur, and therefore no analogy may be drawn from the action of HCG. However, reexamination of the literature (vide Tamura, 1926; Howard, 1927; Deanesly, 1928; Takewaki, 1936; Parkes, 1945) reveals that the general rule is for X zone degeneration to take place between days 7 and 12 of first pregnancy. Implantation in the mouse takes place on day 5 or 6 (Snell, 1946). It may well be that variation in the time of onset of X zone degeneration would, if the facts were available, be found to vary in the same way as time of implantation. Hence the difference between the pseudopregnant and the pregnant mouse in terms of hormones, would lie in an additional foetal-endometrial hormone present in the latter. This acting directly or via the ovary would cause X zone degeneration. More research on this subject may well reveal widely applicable findings on the hormones of pregnancy.

It appears that castration of the adult mouse allows a gonadotrophin to act upon adrenal cortical cells to produce a secondary X zone. In the normal mouse no response may be elicited in the presence of circulating androgens. From the gonadotrophin preparations used there is some indication that LH may be the factor involved, although, as in the case of the primary X zone, FSH or a synergism of the two cannot be excluded. Certainly castration of the rat results in increase in stored LH (Hellbaum and Greep, 1940). Parabiosis experiments with joined castrated male-hypophysectomized male indicate that

some of the LH is secreted as evidenced by the androgen stimulation of the prostate and the seminal vesicles of the hypophysectomized partner (Cutuly, McCullagh and Cutuly, 1937). The chief factors in the evocation of the secondary X zone seem to be a steady secretion of LH and the absence of circulating androgens. The castrate male would appear to fulfill these conditions.

How far these findings in the mouse may be applied to the mammalia as a whole is not known. In the human, the fetal cortex has been thought to be similar to the mouse X zone (Grollman, 1936). The fetal cortex begins to degenerate soon after birth and normally seems to have no physiological role. Under abnormal conditions, in the adreno-genital syndrome, the production of androgens has been attributed to it (see Parkes, 1945). It is probable that both the mouse X zone and the fetal cortex develop embryologically without hormonal influence.[3] In the mouse the X zone which differentiates out embryologically (Waring, 1935) becomes competent to respond to LH. Normally the human fetal cortex degenerates before this is possible. It could be envisaged in the human, that under abnormal conditions the pituitary may produce LH at a much earlier age when the undegenerated fetal cortex was able to respond. This response could be, perhaps, the production of androgens to account for at least one kind of prepuberal adreno-genital syndrome. In the mouse the X zone normally comes under the influence of LH but there is no evidence of androgen production from this zone (Gersh and Grollman, 1939a and 1939b; Howard, 1946). The X zone may be regarded as a potential androgen producing layer which under certain abnormal physiological conditions might be able to turn over to steroid hormone production. The conditions for this are not known but it is interesting to note that the long-term castrated male mouse produces sexogens from adrenal neoplasms (Wooley and Little, 1945a & b).

SUMMARY

Three zones of the mouse adrenal cortex, the zona glomerulosa, the zona fasciculata and the X zone each have a different relationship to the secretions of the anterior pituitary. The zona glomerulosa is capable of an existence independent of the hypophysis but the presence of acetone-soluble sudanophilic material related to hormone activity within the zone depends on adrenocorticotrophin. The zona fasciculata is completely dependent on adrenocorticotrophin. The X zone is dependent on a gonadotrophin, possibly LH in nature. Similarly, the secondary X zone which arises in the postpuberally castrated male mouse, is dependent on a pituitary gonadotrophin, possibly LH in nature.

[3] The absence of the X zone from the dwarf mice of Deanesly (1938) may be due to embryological reasons and not to some postnatal hormone deficiency.

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PERIODIC UTERINE BLEEDING IN OVARIECTOMIZED MONKEYS GIVEN CONSTANT DAILY DOSES OF OESTROGEN

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UTERINE bleeding does not take place during the administration of adequate doses of oestrogen to ovariectomized rhesus monkeys, but only as a withdrawal effect some days after treatment has been stopped. If the dose is inadequate bleeding occurs neither during nor after the end of oestrogen treatment. On the other hand, it takes place periodically during a course of treatment in which the animals are maintained on a constant intermediate level of the hormone (Zuckerman, 1937, 1940), the intervals between successive phases of bleeding being referred to for convenience as "artificial threshold cycles" or as "threshold cycles." This observation bears significantly on explanations of the hormonal control of normal menstrual bleeding.

The present paper shows that a succession of such cycles may be induced over a period of several years, and that they are as readily induced by stilboestrol as by oestrone.

MATERIAL AND METHODS

Ten ovariectomised rhesus monkeys (*Macaca mulatta*) were used in this study. Details about the animals and the experiments are given in Table 1. Daily injections of oestrogen were given intramuscularly in arachis oil solution. Daily vaginal lavages were made to detect the occurrence of uterine bleeding.

In establishing the level of stimulation necessary to cause recurrent phases of bleeding, an arbitrary amount of oestrogen was injected daily, usually for not less than 100 days. If bleeding did not occur during this period, the injections were stopped. If withdrawal bleeding then occurred, the injections were re-started at a lower level. If it did not occur, the daily dose was raised. In either case, the procedure was continued until bleeding occurred during the course of injections.

RESULTS

Oestrone

Threshold cycles were successfully induced in three of seven monkeys treated with oestrone. They occurred occasionally in three others, and not at all in the seventh.

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The experiment was most successful in two young mature monkeys (OM 536, 537) that had not been experimented on before, and in whom treatment was begun within a few weeks of the removal of their ovaries. OM 536 underwent 27 successive cycles in 1088 days on 10 μg. oestrone daily (mean cycle length 40.3 ± 3.93 days) before failing to respond. Four more cycles occurred on daily doses of 20

TABLE 1. DETAILS OF ANIMALS USED

Animal No.	Total amount of oestrogen (mg.)	Days under experiment	Body-wt. at start (g.)	Body-wt. at end (g.)	Previous history
71	7.534	1198	6100	7540	Mature. 43 phases of experimental oestrogenic stimulation.
452	4.891	866	4060	5760	Young mature. No previous treatment.
461	4.388	829	4120	6580	Young mature. No previous treatment.
382	15.640	1600	6320	7400	Mature. 24 phases of experimental oestrogenic stimulation.
69	7.282	697	7750	7280	Mature. 43 phases of experimental oestrogenic stimulation.
533	9.786	916	4160	4140*	Adolescent. 5 phases of experimental oestrogenic stimulation.
536	15.940	1469	3800	7000	Prepubertal. No previous treatment.
537	12.10	1287	3720	8820	Prepubertal. No previous treatment.
447	13.983	1879	2600	5600	Prepubertal. No previous treatment.
430	20.499	1782	4020	8000	Adolescent. 8 phases of experimental oestrogenic stimulation

* The maximum body-weight reached during the experiment was 6780 g. Subsequently, however, the animal lost weight.

μg. and two more on 15 μg. The experiment was ended on the 1469th day.

OM 537 underwent 26 successive cycles in 1112 days on 10 μg. oestrone daily (mean cycle length 42.8 ± 6.10 days) before a period of 89 days' amenorrhoea occurred and the experiment was discontinued.

In the third successful experiment (OM 382) twelve cycles occurred in a period of 303 days on 10 μg. oestrone daily. These were followed by a phase of amenorrhoea which lasted 86 days. The dose of oestrone was then lowered to 8 μg. but no bleeding occurred during the 110 days it was maintained at this level. Bleeding occurred again when the dose was raised to 10 μg. daily (3 cycles in 236 days). The dose was then lowered to 9 μg. daily, for a period of 184 days, in which 6 cycles occurred. In the following 319 days it was kept at 10 μg. and 9 cycles occurred. Withdrawal bleeding followed the cessation of injections (on the 1600th day of the experiment) and the monkey was autopsied 30 days later. The uterus was small, and on micro-

scopic examination the endometrium appeared thin. It contained numerous straight glands embedded in a dense stroma.

The earlier history of two of the three monkeys in which the experiment was partially successful has already been recorded (OM 477 and OM 430, Zuckerman, 1940) OM 447 began with 6 threshold cycles on 10 μg. oestrone daily. A period of 553 days' amenorrhoea followed, which was interrupted by one day's bleeding on the 200th day. During this long phase of amenorrhoea the level of oestrogen treatment was gradually dropped to 7 μg. for 82 days, 5 μg. for 223 days, and 3 μg. for 131 days. Uterine bleeding did not occur when the injections were stopped.

It was thought that the uterus might have become insensitive during this long period of treatment with sub-threshold quantities of oestrogen. The animal was therefore given 100 μg. oestrone daily for 10 days. Withdrawal bleeding started 7 days later. The animal was then put on to 10 μg. oestrone daily. Three threshold cycles occurred, before amenorrhoea once again supervened. Further attempts to establish recurrent threshold cycles on 8 μg., 8.5 μg., 10 μg. and 11 μg. oestrone daily were unsuccessful, and the experiment was ended on the 1879th day.

The first four threshold cycles by OM 430 already recorded occurred at a level of 10 μg. oestrone daily. Amenorrhoea then supervened, but cycles were re-established temporarily on 8 μg. daily. A second period of amenorrhoea lasting 157 days ensued, and subsequent daily doses of 5 μg. (for 63 days) and of 10 μg. (for 65 days) did not induce bleeding. Cycles were re-established at a level of 15 μg., seven occurring during a period of 527 days. 112 days' amenorrhoea followed, presumably due to an excessive amount of oestrogen, since withdrawal bleeding followed the cessation of the daily injections. The experiment was resumed at a daily dose of 13 μg., but bleeding did not occur during the 113 days of the experiment, nor as a withdrawal effect when the injections were again stopped. They were then resumed but threshold cycles could not be re-established either on 14 μg., 12 μg. or again on 15 μg. daily, and the experiment was ended on the 1782nd day.

Only a few threshold cycles were induced in the third monkey (OM 533) in the group of partially successful experiments. One of 65 and one of 96 days occurred during each of two courses of injections of 10 μg. oestrone daily. Both ended with periods of amenorrhoea lasting over 100 days, and neither was followed by withdrawal bleeding. Two threshold cycles of 26 and 31 days were then obtained on 12 μg. daily, and three (22, 26 and 87 days) on 15 μg. before amenorrhoea again set in. Withdrawal bleeding occurred 9 days after the 15 μg. injections were stopped. One further cycle was obtained on 13 μg. daily. The animal was autopsied on the 17th day of the subsequent incomplete cycle. The uterus was normal in size. The endo-

metrium appeared thin and inactive and contained a number of straight glands, in a non-oedematous stroma.

Treatment was continued for a total of 697 days in the one completely unsuccessful experiment (OM 69). Bleeding did not occur at dose levels of 8 μg. (for 119 days), 10 μg. (for 133 days), 15 μg. (for 68 days), and 20 μg. (for 150 days). Bleeding occurred seven days after the 20 μg. injections were stopped. The monkey was autopsied 35 days after the last injection.

The uterus was normal in size. Parts of the endometrial stroma were replaced with fibrous tissue, but in general it was moderately oedematous and contained straight glands in which there were several mitotic figures. The vaginal epithelium was deeply stratified.

Stilboestrol

Three monkeys (OM 71, OM 452, OM 461) were used in attempts to establish threshold cycles of uterine bleeding on constant daily doses of stilboestrol.

In the first (OM 71) six cycles were obtained on 10 μg. daily. Withdrawal bleeding followed the cessation of these injections, and of subsequent courses of injections of 7.5, 5.0, 3.5, 2.5 and 2.0 μg. daily. Two cycles in a period of 89 days were obtained at a level of 1.5 μg. stilboestrol daily. The monkey was autopsied 31 days after the last injection. The uterus was normal in size. The endometrium was thin and there was an exceptionally dense condensation of stroma under the eptihelial lining of the cavum uteri. There were only a few straight glands in which some mitoses could be seen.

The amounts of stilboestrol given to OM 452 were progressively reduced from 10 μg. to 3.5 μg. daily. At this level 10 successive cycles were obtained in a period of 421 days. In the third and last animal (OM 461) a single cycle occurred at an injection level of 10 μg. daily. Cycles did not recur until the dose was reduced to 2.5 μg. daily. At this level 10 successive cycles were obtained in a period of 240 days.

ANALYSIS OF THE RESULTS

Tables 2 and 3 show the mean length of the threshold cycles and the duration of bleeding for the dosages of hormone employed.

The dose of oestrogen necessary to produce threshold cycles is variable and not easy to determine. As previously reported (Zuckerman, 1940), between 5 and 20 μg. are required daily in the case of oestrone. Stilboestrol is three to four times more active. Cycles seem to be established more easily in younger animals with little or no previous experimental oestrogen treatment than in older monkeys whose uteri may have undergone abnormal changes.

There is clearly no systematic relation between the amount of hormone given and the mean cycle length. The wide variation in cycle-length seems to depend on differences in responsiveness be-

TABLE 2. CYCLES OBTAINED WITH THRESHOLD DOSES OF OESTRONS DAILY

Dose of hormone μg. daily	No. of monkeys used	No. of cycles obtained	Mean length of cycles (days) ±s.e.m.	Mean duration of bleeding (days) ±s.e.m.
20	1	5	31.2±5.0	7.2±0.8
15	4	13	57.4±8.2	8.7±0.7
13	1	1	26.0± —	7.0± —
12	1	2	28.0±3.0	6.0± —
11	1	1	27.0± —	2.0± —
10	5	79	42.1±2.9	6.1±0.3
9	1	6	30.7±5.3	5.2±0.7
8	2	7	45.0±7.4	4.6±1.1
5	1	1	23.0± —	4.0± —
All experiments: 115			42.3±2.3	6.2±0.25

tween animals and within an individual animal from time to time. For example, threshold cycles were established at three different times in one monkey (OM 382) at a level of 10 μg. oestrone. In the first experiment the mean length of 12 cycles was 23.2 days, and of the duration of bleeding 5.1 days. The corresponding figures for the second series (4 cycles) were 78.7 and 1.7 days, and for the third (9 cycles) 35.4 and 5.2 days.

TABLE 3. CYCLES OBTAINED WITH THRESHOLD DOSES OF STILBOESTROL DAILY

Dose of hormone μg. daily	No. of monkeys used	No. of cycles obtained	Mean length of cycles (days) ±s.e.m.	Mean duration of bleeding (days) ±s.e.m.
10	2	7	56.3± 9.2	8.0±1.6
3.5	1	10	42.1± 4.5	11.4±2.1
2.5	1	10	24.0± 2.2	8.4±1.0
1.5	1	2	44.5±10.5	2.5±0.5
All experiments: 29			39.5± 3.7	8.9±0.97

The mean length of all the 115 threshold cycles induced by oestrone (42.3±2.3 days) or of the 79 cycles which occurred at the level of 10 μg. oestrone (42.1±2.9 days) is very close to the average figure of 43±2.6 days previously reported for a smaller series of observations (Zuckerman, 1940). The duration of bleeding is, however, shorter (6.2±0.25 days) as compared with 7.0±0.1 days (Zuckerman, 1940). Despite great variation from one monkey to another, the mean length of the stilboestrol cycles (39.5±3.7 days) is also close to the figures already recorded for cycles induced by oestrone, though the duration of bleeding is somewhat longer (8.9±0.97 days). Besides being much more variable, the length of the experimental cycles is considerably greater than in normal menstrual cycles.

DISCUSSION

The present findings confirm the observation that periodic fluctuations in the responsiveness of the uterus occur in the course of constant

daily treatment with oestrogen. Threshold artificial cycles were, for example, obtained without interruption in two monkeys over a period as long as 3 years. It seems reasonable to suppose, therefore, that periodic changes in uterine sensitivity also play a part in the control of the normal menstrual rhythm.

Corresponding fluctuations in sensitivity have been observed in the vagina. They occur in rats treated with a constant daily dose of oestrogen (del Castillo & Calatroni, 1930; Wade & Doisy, 1935; Bourne & Zuckerman, 1941; del Castillo & Di Paola, 1942), and take place in phase with cyclical changes in the size of the adrenal cortical cells (Bourne & Zuckerman, 1941). The fact that the adrenal changes also occur in hypophysectomized animals suggests that the rhythmic changes in the cortex may be primary to changes in the sensitivity of the reproductive tract to oestrogen, or a cause of cyclical variation in the total amount of circulating steroid hormone. Preliminary experiments to test this hypothesis in monkeys (Zuckerman, 1940) were unsuccessful owing to operative mortality.

Gilbert & Gillman (1944) have also reported that cyclical fluctuations occur in the size of the sexual skin of baboons maintained at a constant level of oestrogenic stimulation. Despite the fact that cyclical changes do occur in the adrenal cortex, in at least the rat, in phase with those that occur in the reproductive organs, they have attempted to explain their observation without reference to a possible adrenal mechanism. They suggest that the active sex skin requires more oestrogen to maintain it in a swollen condition than is needed to induce swelling at the start. When this extra amount is lacking, as would occur on a constant level of stimulation, the swelling declines until a store of oestrogen is built up sufficient to restimulate swelling.

The occurrence of uterine bleeding in ovariectomized women during a course of oestrogen injections has sometimes been taken to throw doubt on the accepted view that menstrual bleeding is generally due to an oestrogen-withdrawal effect. Occasional observations reported by Bishop (1938), Reynolds, Kaminester & Schloss (1940), and Di Paola & del Castillo (1942), however, show that threshold artificial cycles of the kind discussed in this paper also occur in women, and resemble those obtained in monkeys in that they are rather irregular and longer than normal.

SUMMARY

Periodic uterine bleeding occurs in spayed monkeys injected daily with constant threshold amounts of oestrone. In two monkeys such threshold cycles have occurred without interruption for as long as three years.

Stilboestrol produces the same effect in doses one-third to one-quarter that of oestrone.

The experimental inter-menstrual period (40–42 days) is longer and more variable than the normal menstrual cycle.

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NOTES AND COMMENTS

ACCELERATED GROWTH WITH SEXUAL PRECOCITY IN FEMALE MONKEYS RECEIVING TESTOSTERONE PROPIONATE[1]

IT HAS been possible to double the growth rate and reduce by half the age of the young female monkey at time of menarche (first menstruation). The androgen, testosterone propionate,[2] was the agent used and the animal, *Macaca mulatta*, or rhesus monkey.

In a rather well-standardized monkey colony of five generations, the age at which the monkeys mature is about two years, when they weigh around 3500 grams. Crystalline testosterone propionate dissolved in vegetable oil was given intramuscularly to two young monkeys weighing 1410 and 1600 grams. The dosage was 7.5 mg. per kilo per week.[3] Hormone treatment began at five months, inducing an immediate rise from the normal curves for body weight and length (see graphs). At one year the treated animals reached the weight and length of two-year-olds and menarche occurred exactly at the end of a year in one animal, and at one year nineteen days in the other. Androgen treatment was then discontinued.

This was a true beginning of sexual maturity and not just a spurious uterine bleeding, for six and seven menstrual cycles, somewhat irregular as typical of adolescence, have followed, accompanied by the external skin changes characteristic of the maturing monkey. This great augmentation of growth and differentiation of tissues has not been analyzed. At present one is inclined to interpret it as a general anabolic effect of the nitrogen retention action of the androgen but the possible release of growth hormone, of increase in hypophyseal hormones, protein in nature, or some other intervening mechanism may contribute. The future of the individual animal and tissues which have been subjected to this great acceleration in growth rate is of interest and possible importance.

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[1] This investigation was supported in part by a research grant from the National Cancer Institute, U. S. Public Health Service.

[2] Oreton, supplied by Schering Corporation.

[3] Earlier work with the male monkey indicated that this dosage might be optimal. van Wagenen, Fed. Proc. 6: 219. 1947

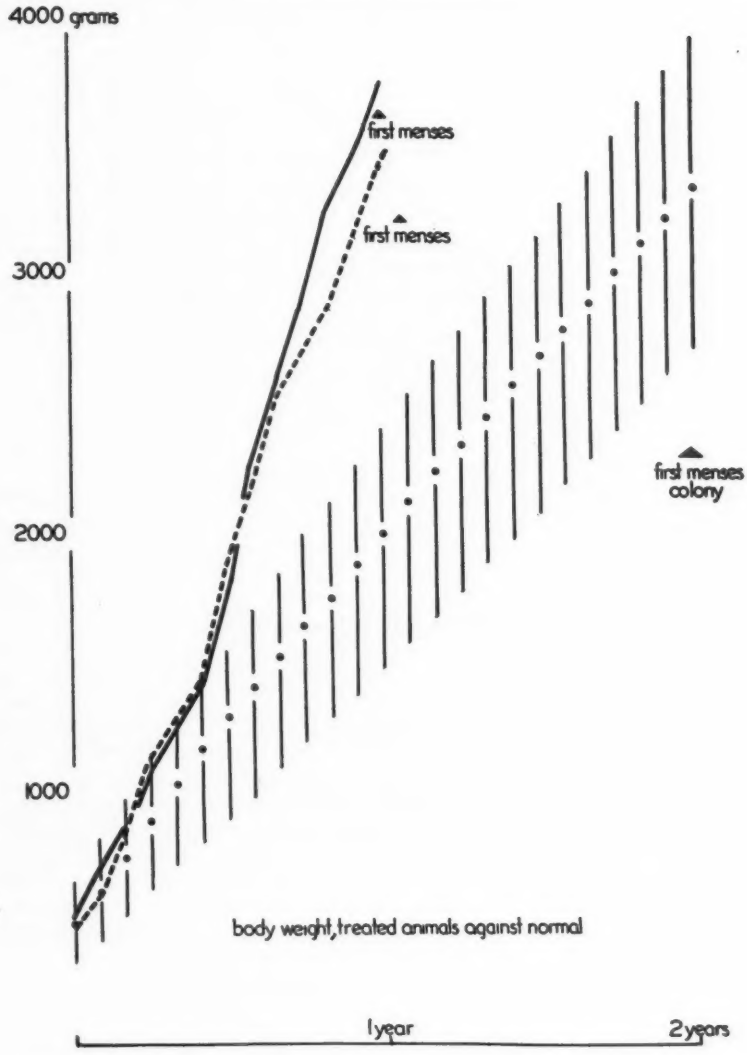

FIG. 1. Individual weight curves of the two hormone-treated animals shown with the span of curves of twenty normal young monkeys recently born and raised in the colony.

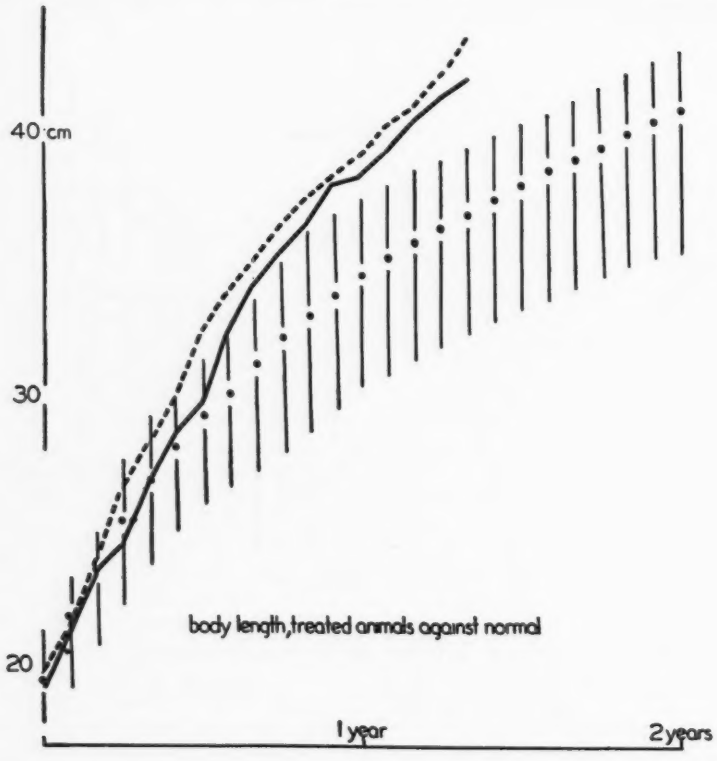

FIG. 2. Body length curves plotted with similar data for the twenty untreated young monkeys. There is a lag in the final effect on body length after a given injection of hormone so the curve is continued beyond the point (one year) where injections were stopped.

ANNOUNCEMENT OF THE 1950 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-Second Annual Meeting of The Association for the Study of Internal Secretions will be held at the Sir Francis Drake Hotel, Friday and Saturday, June 23 and 24, 1950, in San Francisco, California.

The Committee on Local Arrangements is comprised of Dr. Hans Lisser, Chairman and Doctors Leslie L. Bennett, Roberto F. Escamilla, Minnie B. Goldberg, Gilbert S. Gordan, Laurance W. Kinsell.

Hotel accommodations will be difficult to secure on short notice; therefore, members are urged to make their reservations at once. All requests must be addressed to: Dr. William Howard Rustad, American Medical Association (Hotel Committee), Room 200, Civic Auditorium, San Francisco, California.

The scientific sessions will be held in the Empire Room of the Sir Francis Drake, and registration will be on the same floor. The annual dinner will be held in the Empire Room on Friday, June 23 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Edward A. Doisy, M.D., St. Louis University School of Medicine, 1402 South Grand Avenue, St. Louis, Missouri, not later than March 1, 1950. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and journals of the Association.* Names of non-members who are co-authors must be followed by the words "by invitation," and the principal degree of each author.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1950.

Announcing

POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY INCLUDING DIABETES

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The Roney Plaza, one of Miami Beach's most delightful hotels, offers special convention rates to members of this assembly. This is an unusual opportunity for you and your family to enjoy a pleasant vacation and for you to participate in a highly instructive program of the latest advances in endocrinology and metabolism.

A fee of $75 will be charged for the entire course and the attendance will be limited to 100. REGISTRATION WILL BE IN THE ORDER OF CHECKS RECEIVED AND WILL CLOSE ON MARCH 3, 1950. Should there be an insufficient number of applicants to fill the course, the registration fee will be refunded immediately in its full amount.

Application for approval of this course has been made to the Veterans Administration. Veterans should make formal application to their local agencies on the appropriate form (1905e or 1950) as furnished by the V.A.

Please forward application on your letterhead together with check payable to The Association for the Study of Internal Secretions, to Henry H. Turner, M.D., Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma, before March 3, 1950. Further information and program will be furnished upon request.

Hotel reservations should be made directly with the Roney Plaza Hotel, Miami Beach, Florida, and the hotel advised that you are attending this Postgraduate Assembly.